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Review

Affinity capillary electrophoresis: important application areas and some recent developments

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Abstract

Affinity capillary electrophoresis (ACE) is a broad term referring to the separation by capillary electrophoresis of substances that participate in specific or non-specific affinity interactions during electrophoresis. The interacting molecules can be found free in solution or can be immobilized to a solid support. Every ACE mode has advantages and disadvantages. Each can be used for a wide variety of applications. This paper focuses on applications that include purification and concentration of analytes present in diluted solutions or complex matrices, quantitation of analytes based on calibration curves, and estimation of binding constants from direct and derived binding curves based on quantitation of analytes or on analyte migration shifts. A more recent chemicoaffinity strategy in capillary electrophoresis/capillary electrochromatography (CE/CEC) termed molecular imprinting ('plastic antibodies') is discussed as well. Although most ACE studies are aimed at characterizing small-molecular mass analytes such as drugs, hormones, and peptides, some efforts have been pursued to characterize larger biopolymers including proteins, such as immunoglobulins. Examples of affinity interactions that have been studied are antigen–antibody, hapten–antibody, lectin–sugar, drug–protein, and enzyme–substrate complexes using ultraviolet, laser-induced fluorescence, and mass spectrometer detectors. This paper also addresses the critical issue of background electrolyte selection and quantitation of analytes. Specific examples of bioaffinity applications are presented, and the future of ACE in the biomedical field is discussed.

Keywords: Reviews; Affinity capillary electrophoresis; Competitive immunoassays; Analyte concentrator–microreactor device; Molecular imprinting; Preconcentration; Binding constants

Contents

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1. Introduction

The ability to determine the presence of drugs, intermediate metabolites, and biopolymers in body fluids, cells, and tissue specimens is of considerable importance in the biological sciences. The wide range of chemical structures and the complexity in the physiological function of every biomolecule contribute to the problems encountered when trying to analyze these materials. Determining individual analytes in complex biological samples often requires some type of separation as a prerequisite for their measurement. A number of techniques exist for the determination of biomolecules, and every one of these techniques has its special attribute which may include speed of analysis, selectivity, sensitivity, adequate throughput capacity, and in most cases, affordable cost of analysis. Multidimensional separation techniques, including two-dimensional electrophoresis, hyphenated techniques, and immunological methods, have been useful biomedical tools for most of the last four decades (for reviews see Refs. [1–3]). Most recently, capillary electrophoresis (CE) has become another tool with significant potential for the determination of analytes in complex biological matrices.

Capillary electrophoresis, and a family of related techniques, including the utilization of microchips, have experienced an explosive growth in the last decade due to their inherent characteristics, which include high separation selectivity, small sample size requirement, high speed of analysis, high efficiency, excellent mass sensitivity, low reagent consumption, and high recovery (if surface adsorption is minimized). The versatility of this technology makes it suitable for the separation of chemically diverse substances, including ions, drug metabolites, peptide, proteins, oligonucleotides, and DNA. An ever-increasing number of CE methods are being used successfully in analytical, biomedical, pharmaceutical, environmental, food, forensic, and clinical laboratories [4–10].

One of the attractive main features of CE is the capability to simultaneously separate a wide variety of analytes, including analytes that form complexes through molecular interactions. This area of research is known as affinity capillary electrophoresis (ACE). As shown in Fig. 1, ACE may be classified into three modes: (a) nonequilibrium electrophoresis of equilibrated sample mixtures. Receptor and ligand in sample, electrophoresis separation buffer empty of both receptor and ligand; (b) dynamic equilibrium affinity electrophoresis. Sample contains receptor protein, electrophoresis separation buffer contains ligand; and (c) affinity-based CE or CEC separations on immobilized selectors.

2. Assay of reversibly interacting analytes in solution

The mobility of complexed molecules is zero in electrophoretic binding assays using immobilized ligands; therefore interactions are demonstrated with a maximum efficacy. However, immobilization of an interacting molecule may impede or artificially enhance the recognition process in which it participates. Also, the concentration of active molecules present and accessible after immobilization on a surface is not determined easily even though a knowledge of this is required for the estimation of binding constants. Additionally, it may be difficult to regenerate the active surface after binding without changing the concentration and activity of immobil-

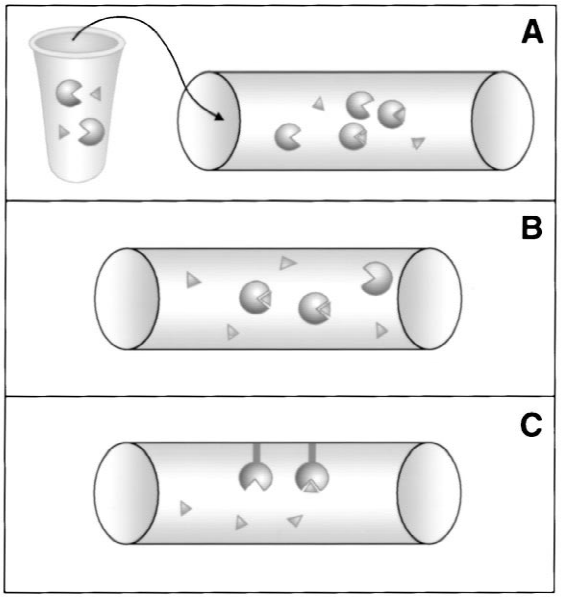

Fig. 1. Schematic diagram of the three main modes of affinity capillary electrophoresis. (A) Nonequilibrium electrophoresis of equilibrated sample mixtures mixed in a test tube. Receptor and ligand in sample, electrophoresis separation buffer empty. An aliquot of the sample is introduced into the capillary for separation. (B) Dynamic equilibrium affinity electrophoresis. Sample contains receptor protein, separation buffer contains ligand. Binding and separation of the affinity components occur within the capillary. (C) Receptor protein is immobilized on the walls of a portion of the capillary, or microbeads, or membranes, or microchannels (see Fig. 13). Ligand present in a diluted solution or a complex mixture is affinity-captured on receptor, nonrelated matrix components are washed away, specific ligand is released from adsorbed state, and separation is performed by capillary electrophoresis.

ized ligand. These issues resemble the problems that may be encountered in surface-binding studies using plasmon resonance analyses [11] and do not normally exist in solution formats.

The basic requirement for the use of solution techniques is that complex formations lead to electrophoretically discernible mobility shifts under native buffer conditions. Electrophoresis can then be used to estimate binding constants from direct and derived binding curves based on quantitation of analytes or based on measurements of migration shifts. Capillary electrophoresis is an attractive method to use for these types of binding assays because of its wide applicability with regards to analytes [4,12–16] and its flexibility with regards to separation conditions. A general advantage of CE, when compared with traditional electrophoresis methods for the study of physiologically important affinity interactions, is the much wider range of buffers that are compatible with the technique, including buffers of physiological ionic strength [17]. Some antigen–antibody reactions are, for example amplified artificially at the low-ionic strength (0.02 *M*) conditions used in traditional electrophoresis techniques [18]. Specific applications of CE-based binding studies of soluble molecules are now quite numerous and have been reviewed in several recent publications [19–23].

Because the electrophoretic mobility of an analyte in CE is roughly proportional to its mass-to-charge ratio [24], there will be situations (e.g., when a small neutral molecule binds to a large receptor analyte) in which the mobilities of complexed and free receptors cannot be discriminated even by CE. Changes in peak appearance times must be at least of the order of the analyte peak width in order to be measured reliably [25]. Charge modifications of the ligand [25] or use of the small ligand as the analyte [26] may in some cases allow binding data to be obtained in such situations, but if this cannot be achieved, the use of soluble reactants for the study of binding is precluded. Also, the rate constants (on-and-off rates) characterizing the interaction have important consequences for the type of electrophoretic binding assay to use. Alternative methods have been devised when binding kinetics are incompatible with the analysis of preequilibrated mixtures. The methods are reviewed below, with an emphasis placed on the influence of the rate constants on the experimental approaches and on applications of CE-immunoassays and binding constant determinations. Discussion of the methods will be divided on the basis of the contents of the samples and of the electrophoresis buffer.

2.1. Non-equilibrium electrophoresis of equilibrated sample mixtures: receptor and ligand in sample, electrophoresis separation buffer empty

The approach is illustrated in Fig. 2. Samples are analyzed after equilibration of mixtures consisting of receptor and ligand at different ratios. In this case, electrophoresis is simply a tool to separate and

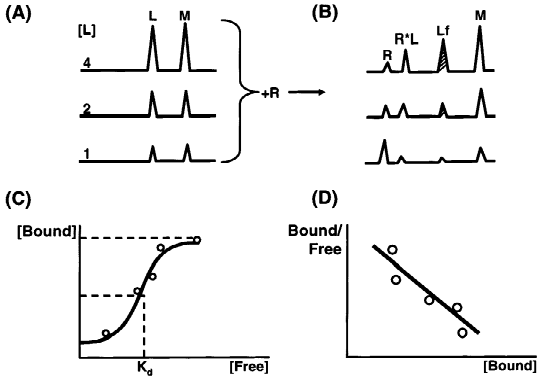

Fig. 2. Principle of CE-based binding studies of stable pre-equilibrated complexes. (A) Generation of data for calibration curve of peak area as a function of concentration of ligand (L). A marker molecule (M) is included in each incubation. (B) Experiment under same conditions as in (A), but samples are pre-equilibrated with a fixed concentration of receptor (R) before electrophoresis. Area of free ligand (Lf) after incubation is used to obtain amount of bound ligand in each analysis of incubated samples. (C) Data treatment (direct binding curve, yields K_d as the Lf concentration at half-saturation). (D) Scatchard plot, slope is $-1/K_d$.

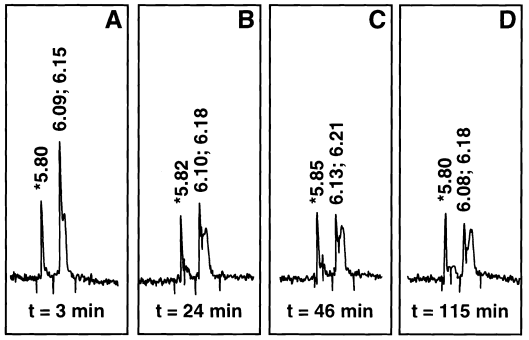

Fig. 3. Determination by CE of equilibration time for a binding interaction. Repeated 2-s (1.8-nl) injections of a mixture of 5 μl, 20 mg/ml heparin, and 20 μl 0.22 mg/ml (145 m*M*) of the synthetic heparin-binding peptide [116] CH_3CO–Ser–Ile–Arg–Gly–Tyr–Val–Ile–Ile–Lys–Pro–Leu–Val–Trp–NH_2 and an impurity (*) in 10% (v/v) dimethylsulfoxide. Samples were separated using a 50-μm I.D. uncoated fused-silica capillary filled with 0.1 *M* phosphate, pH 7.4, at 20 kV. Detection was at 200 nm, and total length of the capillary was 57 cm, with 50 cm to the detector window. Incubation times are given in the figure.

quantitate bound and free molecules. The on-line detection methods used in CE are well suited for this, as opposed to the indirect and imprecise methods of quantitation possible in traditional gel electrophoresis. Peaks corresponding to free ligand in a series of incubation mixtures are integrated, and binding curves of bound ligand as a function of free or total offered or the bound/free ratio as a function of bound ligand can be constructed subsequently on the basis of calibration curves of known amounts of ligand [27–30]. Binding stoichiometry can be extracted from the binding curves or by plotting the data as described elsewhere [31]. Although the approach bears much resemblance to commonly used binding assays that separate preincubated mixtures of molecules according to size such as equilibrium dialysis, size exclusion chromatography, and filtration, the use of CE offers at least three immediate advantages: (a) the receptor molecule does not necessarily have to be pure [32], (b) there is no requirement for size differences between the ligand and the receptor molecule and, (c) the time necessary for reaching equilibrium in the sample mixture is easily followed by repeated CE analyses of sample aliquots, because typically only nanoliter amounts are consumed in each analysis and because samples can be kept at a constant temperature in many instruments [26]. Fig. 3 shows the monitoring of binding in a 20 μl sample mixture from which aliquots were injected at the times indicated. The area of the peak corresponding to the free peptide decreases with time.

As in many types of gel electrophoretic binding assays, for example, gel retardation assays for DNA-binding proteins [33,34], analysis of binding using equilibrated sample mixtures is suited only for strong interactions. The limiting factor is the dissociation rate of complexed molecules. Complexes formed during the incubation must be quite stable so that a subsequent quantitation of the free ligand peak reflects the concentration of free ligand in the sample without contributions from complexes that dissociate during the introduction and movement of the sample into the empty electrophoresis buffer [34]. It follows that it will be impossible to detect any interactions by this method if the dissociation rate is too rapid. If no more than 10% of specifically bound ligand is to be permitted to dissociate during the separation, the dissociation rate constant (k_{off}) must be less than $0.105/t$, where t is the analyte separation time [29]. The analyte separation time (t) is not the total analysis time, but rather the time required to intro-

duce the sample and to separate a complex peak from a peak representing free receptor or ligand, or in other words, the time after which dissociating material cannot add to the quantity of free molecules present in the mixture before electrophoresis. Thus, the speed and efficiency of the separation determine the lower limit of affinity that can be characterized by this method.

Another specific limitation of the described use of CE is the relative insensitivity of UV-based detection systems. Estimation of binding constants for tightly interacting species requires the quantitative determination of the fraction of complexed species at receptor concentrations that span the value of the dissociation constant [35], for example, at the non-saturated end of the binding curve. Because the concentration detection limits are rather high with UV absorbance detection (e.g., μM for peptides [27]), strong interactions may be difficult to quantitate. Thus, the use of equilibrated mixtures for CE-based binding constant determination has limitations for both strong (a detection problem) and weak interactions (a time and efficiency problem).

The problems associated with detection limits can be alleviated by using laser-induced fluorescence (LIF) detection [36,37]. This technique results in quite sensitive measurements as illustrated, for example, by assays for DNA-binding proteins in single sea urchin eggs [32] and especially by the many CE-based immunoassays that have been used to determine and quantitate small analytes of clinical interest contained in complex matrices [15,38] and to determine antigen–antibody binding constants [28]. CE immunoassays employing LIF detection for the determination of one or more specific analytes can be carried out as noncompetitive or competitive assays, as outlined in Fig. 4. In the noncompetitive approach, a labeled antibody or a fragment thereof is allowed to react with a sample (Fig. 4A). Binding to a sample component is demonstrated by ensuing changes in the position of the labeled antibody peak in the electropherogram caused by stable complexation of the antibody with its antigen [39–41]. It follows that this approach is particularly well suited for larger and/or charged antigens that are likely to induce migration shifts of the antibody molecule when it binds. In chosen cases, isoelectric focusing can be used to concentrate the analytes; this approach has allowed pM detection limits [40]. The drawback of the method is that because of antibody microheterogeneity, even highly purified monoclonal antibodies will yield a heterogeneous collection of peaks representing labeled antibody molecules. It may therefore be an advantage to work with antibody fragments [39,40]. Also, the quantitation based on area measurements of broad and asymmetric antibody peaks may be imprecise (immunoglobulin molecular mass is approximately 150 000 with a pI usually >6 [25], so capillary wall interactions often are seen at neutral pH [42]). Fluorescence quenching or enhancement brought about by antigen binding may be a further source of error in using the signal of the labeled peak for quantitation, and multiple reacting components in the sample will give multiple peaks. A lack of mobility shift, as seen when binding

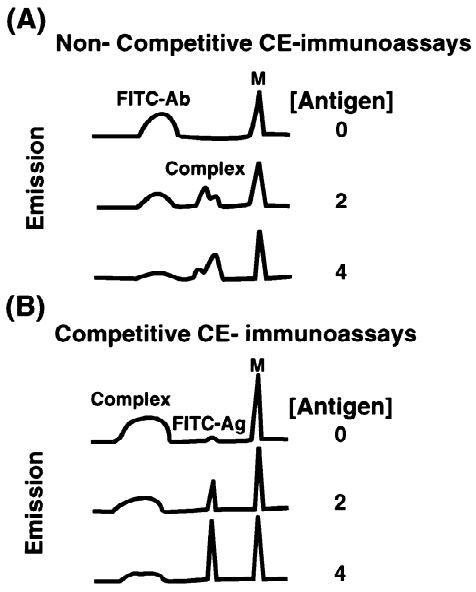

Fig. 4. Principles of CE-based noncompetitive (A) and competitive (B) immunoassays with labeled reagents for measurement of specific analytes using laser-induced fluorescence detection. (A) Fluoresceinylated antibody (FITC–Ab) and fluorescing marker molecule (M) mixed with increasing amounts of unlabeled antigen (Ag) of low mobility. Complex peaks are double-peaked, corresponding to mono- and bivalent antibody occupancy. (B) Fluoresceinylated antigen (FITC–Ag) and marker (M) are mixed with antibody and with a sample containing increasing amounts of unlabeled antigen that displace FITC–Ag from the antibody–FITC–Ag complex (Complex). Quantitation of unlabeled antigen is based on the area of the resulting FITC–Ag peak.

to a similarly charged molecule such as IgA, may be overcome by modifying the charge of the antibody, for example, by succinylation, to give it a sufficiently different migration from its ligand [38]. However, it must be ensured that manipulations such as these do not influence the reactivity of the antibody.

Competitive immunoassays (Fig. 4B) avoid most of the above-mentioned problems. These assays have been used mainly to determine low-molecular weight analytes such as drugs and hormones in complex biological matrices (including plasma, urine, cytoplasm and brain tissue extracts) [43–48] and use a fluorescently labeled antigen as a tracer ligand that is more or less competed away from the antibody by antigen in the sample. Thus, the area of the fluorescent antigen peak, obtained by CE of preequilibrated samples is directly proportional to the concentration of antigen in the sample. In theory, it is also possible to quantitate the analyte on the basis of the area of the complex peak but quantitation of the free tracer peak relative to an internal standard has been found to give the most consistent results [43]. Antigen–antibody complex formation normally is fast and stable, thus the CE step serves simply to separate the free labeled tracer from complexed molecules. As a result, optimized assays may require only a few minutes to carry out [43] and have detection limits in the 0.1-n*M* range [44], depending on the calibration sensitivity and the binding constant for the antigen–antibody interaction [43]. This concentration range does not yet compete with concentration detection limits in traditional immunoassays. The time and amount of material needed for the assay may be minimized further by the application of microchip formats [49]. Interactions with the inner capillary walls are of no consequence in the competitive assays as long as this binding does not affect the antigen binding. Further, more than one analyte may be determined simultaneously [45], and it is generally advantageous to work with single, well-defined labeled ligands instead of heterogeneous proteins. Further developments within this field may involve the use of more sensitive detector systems and systems including mass spectrometry (MS) [50–52] and nuclear magnetic resonance (NMR) [53] that can yield direct information concerning the molecular composition of specific analyte peaks.

Even though binding studies with samples of equilibrated mixtures generally are useful only for strong interactions, lower-affinity interactions may be studied by using the frontal analysis approach, which is a variant of the method used when conditions during electrophoresis are close to equilibrium. The method is equivalent to large zone gel filtration [35] and has been used to characterize drug–protein interactions [54–57] or other rapidly interacting molecules [58] when the supply of materials is not limiting. Large plugs (typically at least ten times the volumes used in zone electrophoresis) of equilibrated samples containing receptor and ligand molecules are introduced into the capillary. The components contained in this large sample plug are in equilibrium during the separation, and the analyte amounts are such that peak plateaus, having heights that are proportional to the analyte concentrations are formed. Frontal electrophoresis, in contrast to zone electrophoresis, relies on the separation of analyte fronts. Solutes are not separated clearly but make up fronts of different concentrations [59]. Thus, the free ligand will generate its own plateau at the front or rear of the receptor–ligand plateau if the mobilities of the receptor molecule and the receptor–ligand complex are equal but different from the mobility of the free ligand [58]. The free ligand and the binding can thereby be quantitated after preincubation of the receptor with various amounts of ligand as in any other preequilibration approach. The method requires that no sample components other than the ligand and the receptor give a detector response; otherwise, contributions from other sample components require correction [58]. The detector sensitivity may again be a problem because precise measurements of small differences in plateau heights can be difficult and because detection limits may allow only partial binding curves to be obtained [58]. The capillary frontal electrophoresis method has been used to study interactions characterized by m*M* dissociation constants [54,56,58].

A further specialized form of the frontal analysis approach included here for completeness is the vacancy peak method [54], in which the whole capillary is filled with a preequilibrated protein–ligand mixture and the sample consists of empty buffer that is introduced into the capillary as a small plug. The buffer plug forms a negative peak (if

analytes give a detector response). The analytes will be mixed in this zone during electrophoresis because of their differential mobilities, and the resulting electropherogram will consist of two negative peaks representing levels of bound and free ligand [54,60]. This method also requires quite large amounts of reactants. It has not been used much in CE-based binding assays and in one study was found to be less suitable than the frontal analysis approach for the study of protein–drug interactions [54].

2.2. Dynamic equilibrium affinity electrophoresis: sample contains receptor molecule, electrophoresis buffer contains ligand

This method for investigating binding interactions by CE (Fig. 5) is the classical affinity electrophoresis approach [61–63] resembling affinity chromatography, in which a receptor molecule is passed through a bed of immobilized ligand. In CE (and in conventional electrophoresis [64]), the ligand may be free in solution and its concentration therefore easily controlled. The presence of an interacting ligand in the buffer during electrophoresis adds an extra dimension to the separation process. The increase in selectivity brought about by this approach is illustrated clearly by the many separations of enantiomeric molecules that can be performed by inclusion of chiral selectors in the electrophoresis separation buffers [65]. In this type of affinity CE, gradual receptor peak migration shifts, as a function of the concentration of ligand in the separation buffer, can be used to derive binding constants for the interaction if the rate constants are suitable (Fig. 5A). The equation linking mobility shifts ($\Delta\mu$) with ligand concentration [L] and K_d is: $\Delta\mu_{max} - K_d\ (\Delta\mu/[L])$. Since $\mu = l/Et$, where l is the migration distance to the detector, E is the field strength, and t the peak appearance time, the equation may be transformed into the practical form shown in Fig. 5B, in experiments using the same E and l, and correcting t-values as shown by t_m-values from a non-interacting marker molecule [23,79]. In contrast to the previous methods, data here are obtained from the electro-

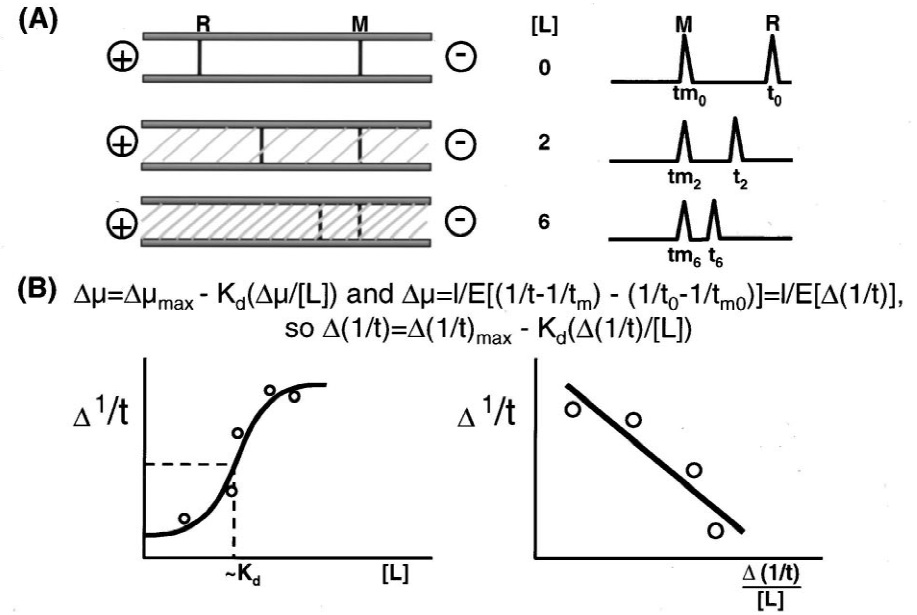

Fig. 5. Principle of CE-based binding studies of quickly equilibrating complexes. (A) Electrophoresis of a receptor (R) and a marker molecule (M) is carried out at increasing concentrations of ligand (L). (B) Based on peak appearance times (t) in the electropherograms and the equations given (where μ is the electrophoretic mobility, l is the length of the capillary from inlet to detector site, and E is field strength) [79], the dissociation constant is estimated from either the direct plot as half the 'saturating' concentration of L or the linearized plot that has ($-K_d$) as the slope.

phoretic pattern alone (i.e., from changes in peak appearance times) [66]. The receptor molecules still exist only in two electrophoretically distinct states – bound or free – but they spend different amounts of time in either state during the time of the electrophoresis experiment, depending on the ligand concentration. A number of analyses must be performed to obtain data points for binding curves, and consequently, the consumption of ligand – even though a capillary volume typically is of the order of 1 to 2 μl – may be considerably higher than in the pre-equilibration method.

To use the approach for valid binding constant determinations, it is required that the value of $1/k_{off}$ be much shorter than the receptor peak appearance time [19,67]; a rule of thumb is that the complex dissociation half-time, expressed as $\ln 2/k_{off,}$ must be less than 1% of the peak appearance time [68]. Changes from the symmetrical and narrow peak shapes that are found in experiments without ligand will be observed if not all receptor molecules spend the same fraction of time in complexes with ligand during the experiment. In this case, broadened, undetectable, or split peaks are observed [42]; this occurs because the on-and-off rates are too slow to ensure the establishment of a dynamic equilibrium. Whereas receptor molecules at saturating concentrations of ligand will have a resulting peak appearance time that approaches the mobility of the receptor–ligand complex itself, the receptor molecules at intermediate ligand concentrations spend only a fraction of their time (corresponding to their fractional saturation) in complexes. In extreme cases with very stable complexes, receptor molecules may split up into two peaks corresponding to a free and complexed fraction [69]. It follows that peak broadening as a result of slow dissociation can be counteracted by increasing the electrophoresis time to allow more time for equilibration [67]. In chosen cases, intermediate peak shape changes may be used to model rate constants and thereby yield binding constants for the interaction [69,70], but generally they make the approach less suited for affinity constant determinations. In addition to a suitable interaction kinetics, the most important assumptions of the equations linking the migration shifts as a function of ligand concentrations to binding constants are the following [67,69]: (a) The concentration of ligand in the electrophoresis separation buffer is much greater than the concentration of receptor in the sample, (b) the binding stoichiometry is 1:1, (c) binding sites are homogeneous and are distributed homogeneously, (d) molecules do not interact with the inner capillary walls, and (e) the presence of an electrical field does not influence the binding behavior. This type of affinity electrophoresis has been used in native gel electrophoresis for some time [63,71–76], but its applicability has increased considerably with its adaptation to the CE format [19,20,77,78]. A specific advantage of this approach is that a knowledge of the precise value of the receptor concentration is not required as long as it is estimated to be lower than the ligand concentration (preferably less than $0.1\times K_d$) [66], and there is no absolute need for a purified receptor preparation. In a number of cases, when the on-and-off rates were suitable and the other requirements mentioned above were fulfilled, the binding constants that are derived from the equations given in Fig. 5 have been validated by independent methods [11,17,70,77–82] and span a range from less than micromolar to molar dissociation constants [21,83].

True equilibrium conditions are in principle obtained only when the preincubation approach discussed in the first section above is combined with affinity CE, with ligand added to the buffer as described here. In this case, the preincubated sample and the separation buffer contain identical total ligand concentrations. Samples also contain the receptor at different concentrations. This approach is equivalent to the Hummel-Dreyer method for the measurement of protein-binding by equilibrium gel filtration [84], but the electrophoretic method is not limited to low-molecular weight ligands interacting with macromolecules [31]. The separation of the equilibrated sample mixture results in an increased ligand concentration relative to the concentration of ligand in the electrophoresis separation buffer at the position where the complex peak appears. Further, a decrease in ligand concentration will appear corresponding to the migration of free ligand because a fraction of it has been removed by complex formation. The area of the trough of decreased ligand concentration is a measure of the amount of ligand bound and may yield quantitative information on the interaction after a series of experiments [54].

The consumption of ligand in this approach is high, and the method requires that the ligand dis-

plays UV absorbance or is otherwise detectable. The method is a true equilibrium approach because there is no change in the total ligand concentration in the vicinity of the receptor molecule during sample equilibration and electrophoresis. The areas of the peak and the trough ideally (and when the receptor molecule displays no absorbance) are identical and equal to the amount of bound ligand [29]. Under saturating conditions, the system can be used to calculate binding stoichiometries [31]. However, the method has not been used much in CE-based binding studies probably because of the consumption of reactants, difficulties in interpretation of data, the existence of other CE methods for quickly equilibrating interactions and the reported imprecision of the data [54].

3. Affinity-based CE or CEC separations on immobilized selectors

These types of separations should be termed electrokinetic affinity chromatography or capillary electroaffinity chromatography performed with CE instrumentation [85].

3.1. Immobilized proteins as affinity selectors in CE

3.1.1. Albumin

Albumin, a transport protein in serum, has been studied extensively for its drug-binding properties [86]. Serum albumin also has been used as a selector for affinity-based chiral separations in liquid chromatography [87–89]. In the same manner, isolated proteolytic fragments of serum albumin have been used in chiral affinity separations [90,91]. Although there was a good qualitative understanding of the requirement for binding to the albumin sites, it was not until the three-dimensional structure of serum albumin was elucidated [92] that its binding behavior at the molecular level was properly understood.

In Nilsson's laboratory, different haptens were immobilized on serum albumin by utilizing glutaraldehyde-coupling procedures for achieving proper antigens and then raising polyclonal antibodies in rabbits. During the coupling procedure, a protein-gel was achieved repeatedly. This coupling protocol was later transferred and performed in CE capillaries. By timing the filling procedure, the gel formation in the detection window was avoided. A cross-linked protein gel was created inside the capillary as a stationary phase without any disturbance of UV-absorbing protein in the detection area. Different types of chiral drugs were resolved successfully with high efficiency [93,94] (See also Fig. 6A and B on this type of affinity-based chiral separation).

At that time, we termed this separation methodology capillary affinity gel electrophoresis (CAGE) [95]. In parallel, but without connection, Baba et al. [96] also used the term CAGE for a similar approach, although they did not create an affinity stationary phase consisting of the selector itself. Instead, they obtained impressive results by using poly(9-vinyladenine) as a macroligand entrapped within a cross-linked polyacrylamide gel for base-specific separation of oligonucleotides. Studies of affinity systems, in which the selector first has been immobilized to a support (silica) and then packed into the capillary column before the affinity separation [97], have been performed, although with less efficiency than described above.

3.1.2. Cellobiohydrolase

Cellobiohydrolase I (CBH I) was utilized as an immobilized affinity selector in the CAGE mode to resolve different chiral β-adrenergic blockers [98,99]. Because CBH I has not enough free accessible primary amines to form a stable gel-network during cross-linking experiments with glutaraldehyde, a different strategy was chosen. To achieve gelation properties, serum albumin was added. Approximately one albumin molecule was used to coordinate four CBH molecules, which formed a stable mixed-protein-gel network. Successful chiral separations of different β-adrenergic blockers on immobilized CBH were achieved as shown in Fig. 7A, B and Fig. 8. No chiral separation of tryptophan or kynurenine was achieved on the co-immobilized serum albumin. The reason for this could be that too few chiral selectors were present to resolve the amount of molecules needed to be seen by UV-absorption detection. Alternatively, the selective cavities [92] in serum albumin were blocked somehow by neighboring CBH molecules in the glutaraldehyde-formed network, or anti-cooperative interaction with the chiral solutes. Attempts with equimolar amounts of chiral selectors, that is, serum al-

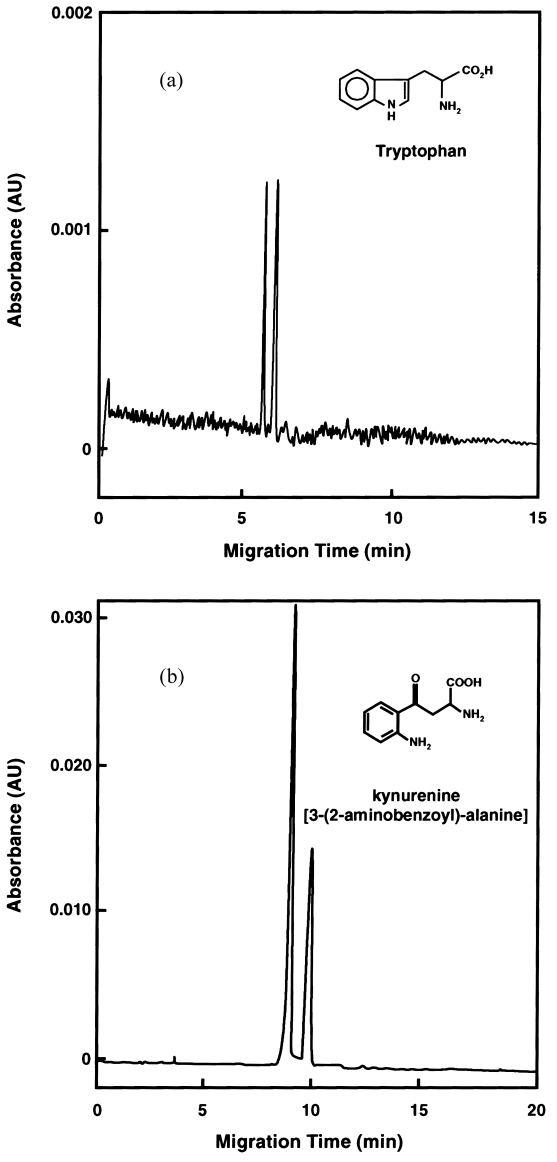

Fig. 6. (A) Capillary affinity gel separation of 70 μ*M rac*-tryptophan on bovine serum albumin gel. Conditions: constant applied field 3 kV (~128 V/cm), 60 μA; gel length=16.5 cm, total length=23.5 cm; run buffer 50 m*M* potassium phosphate, pH 8.2, sample buffer 20 m*M*, pH 6.4; sample injection 3 kV, 3 s. (B) Separation of 250 μ*M* enantiomeric kynurenin. Conditions: constant applied field 3.5kV (~150 V/cm), 67 μA, otherwise as described above.

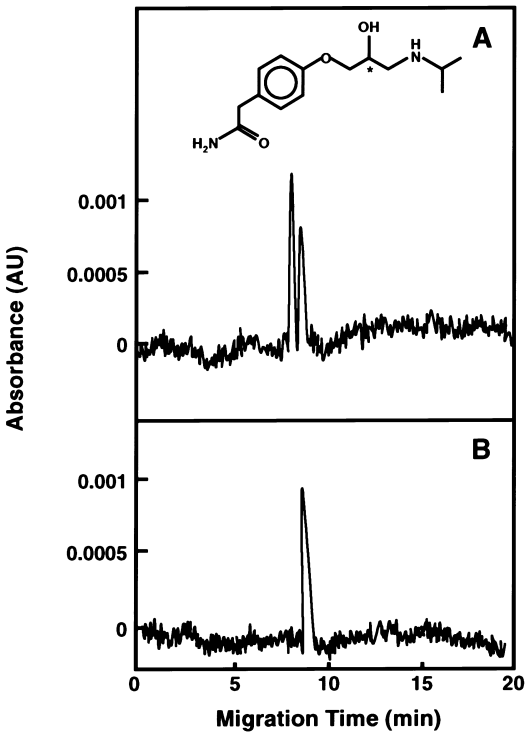

Fig. 7. Capillary affinity gel electrophoretic separation of *rac*-metoprolol with cellulase/BSA gel. (A) 0.15 m*M rac*-metoprolol. (B) 0.075 m*M S*-metoprolol (identification of racemate). Conditions: constant applied electric 3 kV field (~130 V/cm), 40 μA; gel length=16.5 cm, total length=23.5 cm; buffer 50 m*M* potassium phosphate, pH 6.8, with 1% (v/v) 2-propanol; sample injection 2 kV, 3 s.

bumin and CBH, to form a composite gel network, in order to separate two different substance classes on the same CAGE-column, should be performed. As the three-dimensional (3D) structures of both serum albumin [92] and cellobiohydrolase are known currently [100], the molecular mechanism underlying the separation of different chiral molecules can be elucidated. An easier, more convenient, and fully automated process was developed later to create CAGE columns directly in the CE instrument for overnight use which could be ready for use the next day [99].

3.1.3. Monoclonal antibodies

Monoclonal antibodies can circumvent the trial-and-error process of finding a certain proper affinity ligand for a given separation objective. The natural choice is to rely on ‘mother nature’s’ capability of creating specific selectors, so-called ‘biological imprinting’ via the mammalian immune system. After

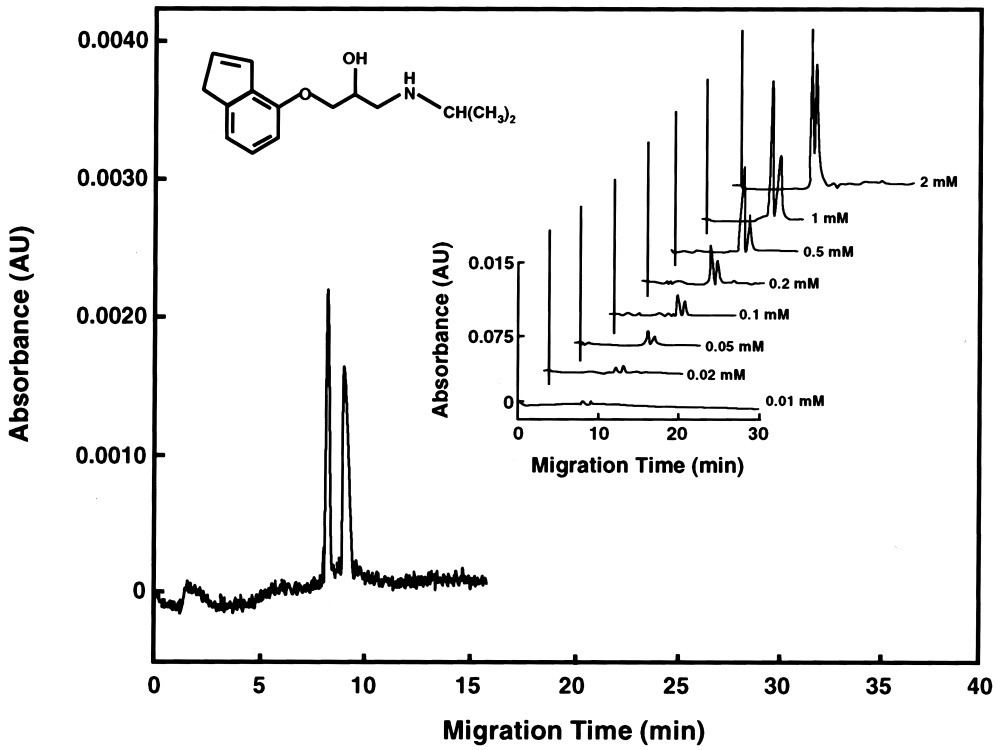

Fig. 8. Capillary affinity gel electrophoretic separation of 0.1 m*M* *rac*-pindolol with cellulase/BSA gel. Inset: Sample loading capacity 0.01–2 m*M*. Conditions: constant applied electric field 3.5 kV (~150 V/cm), 45 μA; gel length=16.5 cm, total length=23.5 cm; buffer 50 m*M* potassium phosphate, pH 6.8, with 1 (v/v) 2-propanol; injection 2 kV, 3 s.

immunization with the proper antigen, spleen cells are isolated, fused with a cancer cell line, and screened for the appropriate selection of cells producing the desired antibody. Isolated cell lines are then capable of in vitro production of the requested selector.

Low binding monoclonal antibodies were selected against the hapten $(Glc)_4$=Glcα1→6Glcα1→4Glcα6→4Glc, and separation was utilized in CAGE format, capable of discriminating between anomeric sugars [101].

3.2. Affinity selectors in CE/CEC created by molecular imprinting

To be our own masters in creating selectors or recognition systems, the concept of molecular imprinting [102–105] was tested in capillary column systems [99,106,107]. The concept of molecular imprinting is to form a synthetic cavity around the analyte of interest where by the cavity will be complementary to the analyte in terms of shape and chemical functionality, that is, to form a synthetic memory with selectivity towards the analyte molecule (‘plastic antibody’). The orientation of functional monomers around the imprinted molecule (the analyte) is essential before the polymerization process. The noncovalent molecular imprinting polymer (MIP) methodology is best performed in organic solvents that are as aprotic as possible because the recognition is based on noncovalent interactions, for example, hydrogen bonding and ionic interactions. In this way the solvent does not disturb the interactions between the functional monomers and the imprinted molecule. The spatial position of the functional monomers in the final polymer matrix is determined by their interaction with the imprinted species (Fig. 9A). After polymerization and removal of the imprinted molecule, the predetermined affinity selectors are ready for use (Fig. 9B).

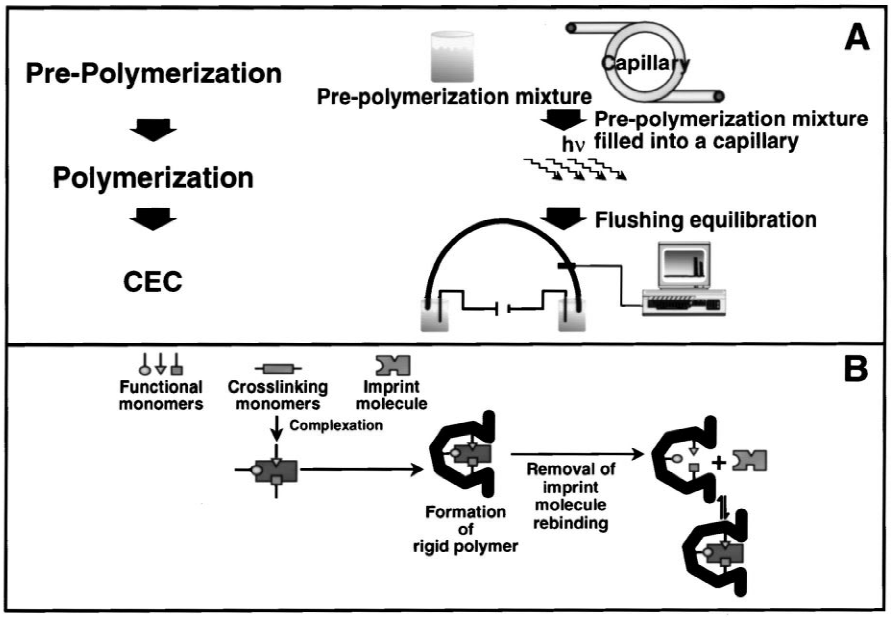

Fig. 9. Schematic representation of imprint formation in capillary columns. (A) A mixture of functional monomer, crosslinking monomer, and print molecule (the analyte) is prepared. As a result of noncovalent interactions between complementary chemical functionalities, the functional monomers come to be arranged around the imprint molecule. (B) After polymerization, the imprint molecule is removed by extraction, exposing recognition sites complementary in size, shape, and chemical functionality to the imprint molecule.

Four different strategies for creating noncovalent molecular imprints within capillary columns have been tested. First, MIPs were created under optimal conditions in a test tube, dried, and then ground into fine particles. The MIP particles were then, in a second polymerization, copolymerized in a hydrogel within the capillary column. This methodology was proven unsuccessful, mainly because of the use of uneven polymer rods, in which air bubbles were trapped easily ruining the capillary electrochromatography (CEC) column.

The second approach was to use MIP fine particles as electrolyte additive. This approach was not successful either, because no chiral separations were achieved and very noisy baselines during CEC were recorded.

The third approach relied on direct photo initiated polymerization of the MIP inside the capillary. To achieve good flow properties, the polymerization process was interrupted before completion, and an imprinted polymer monolith containing superpores of 2–10 μm in width, combined with macropores of approximately 100–300 Å, were achieved (Fig. 10). An on-column detection window was prepared easily by covering a part of the capillary, thus preventing any polymer to be formed in this area. This approach gave chiral separations and the possibility to derive MIP-based columns in less than 3 h.

The fourth concept, related closely to the third, also was performed with direct polymerization in the capillary column. The porous characteristic of the resultant MIP monolith was achieved by using a porogenic agent compatible with the imprinting process. This approach also gave chiral separations, and one advantage was that careful timing of the polymerization reaction was not needed because the reaction could go to completion.

The third and fourth approaches to create MIP-based affinity interaction systems in capillary columns were successful. By making imprints of one optical isomer of a chiral compound, baseline separation of a racemic mixture often resulted, many with acceptable performance and separation time (see Fig. 11), all with predetermined selectivity and thereby known elution order of the enantiomers.

The MIP concept also has been tried in elec-

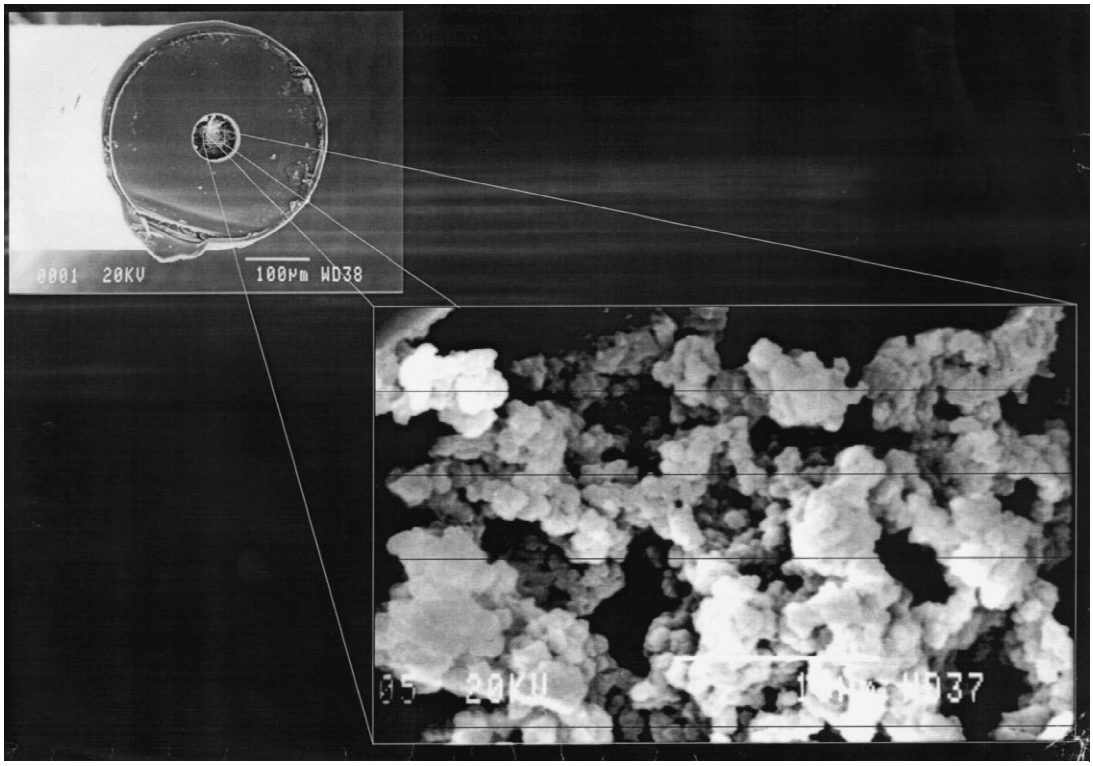

Fig. 10. Electron micrographs of a cross-section of a capillary containing molecularly imprinted polymer. The monolithic polymer can be seen as macroporous micron-sized globules, forming aggregates. The interconnecting superpores (1–20 μm I.D.) are responsible for the low flow resistance through the polymer, allowing the necessary exchange of polymerization solvent to electrolyte as well as easy regeneration of the capillary by hydrodynamic pumping.

troseparation systems by other groups. The use of a dispersion polymerization method to produce capillary columns containing MIP agglomerates has been demonstrated [108]. However, no enantiomer separations could be obtained. Other approaches reported were to introduce MIP particles into a capillary by conventional packing [109] or incorporation in an acrylamide gel [110]. Another approach was to produce an in situ-prepared MIP monolith, in which a conducting agent was present during the polymerization enabling the substitution of the solvent of polymerization for a CEC electrolyte [111,112]. In the latter case, an open capillary had to be connected to the MIP capillary to perform on-line detection. These two methods reported elegantly achieved enantiomeric separations of amino acids. Successful separation of enantiomers in CEC also was reported using capillary columns containing MIP particles, prepared from a chiral functional monomer and used as an electrolyte additive [113].

An interesting and inherent feature of the MIPs is that some cross-selectivity (Fig. 12) can occur between the imprinted molecule (the analyte) and a compound whose structure is closely related. This can be seen as a drawback during predetermined based separation, although, to our knowledge, the imprinted molecule will always be retained the most in the affinity phase created. However, the specificity can be tuned to some degree during the recognition and cross-linking phase. The cross-selectivity can also be used in favorable ways, meaning that the MIP cavity can be seen as an artificial receptor, that is, an analyte molecule can be used to create a receptor mimic. A practical consequence of this is the use of a known drug, to create a receptor mimic, and then to try to find compounds with similar or

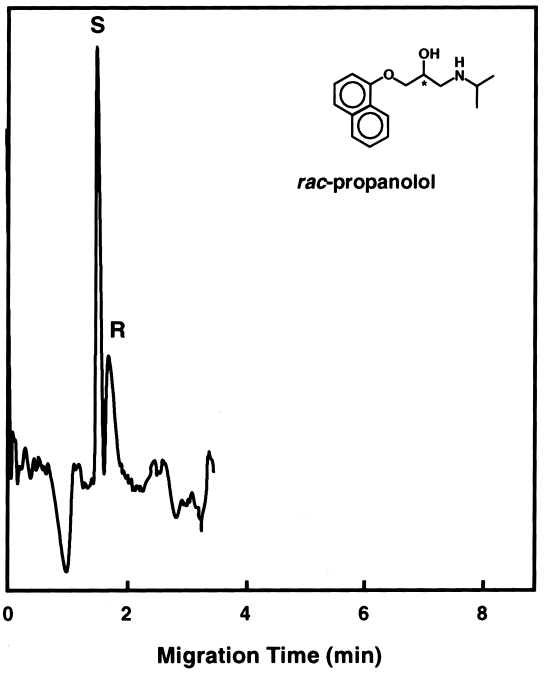

Fig. 11. Predetermined affinity separation of *rac*-propanolol on a capillary column imprinted with the *R*-enantiomer. The sample (100 μ*M*) was introduced electrokinetically into the capillary and separated at a voltage of 30 kV at 60°C and an over-pressure of 7 bar. Running electrolyte; acetonitrile (80%) and 4 *M* acetic acid titrated to pH 3.0 with 4 *M* ammonium acetate (20%).

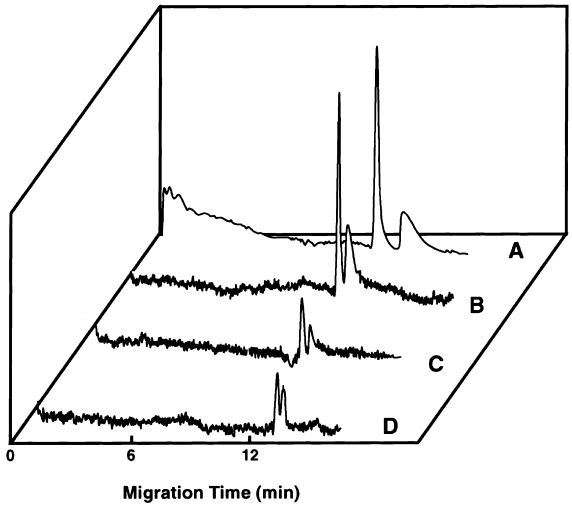

Fig. 12. Predetermined affinity separation of 100 m*M* *rac*-propanolol on a capillary column (90 cm) imprinted with *R*-propanolol, injected electrokinetically (15 kV, 10 s), and separated at 30 kV (333 V/cm) UV absorbance detection 214 nm (A). Separation of *rac*-pindolol as above but UV absorbance detection 280 nm (B). Separation of *rac*-atenolol as above (C). Separation of *rac*-prenalterol as above (D).

more favorable interaction characteristics in experiments, as shown in Fig. 12, in which the β-blockers interact in a way related to how propanolol, from which the receptor-mimic is created, interacts. A new era in drug discovery is revealed.

4. Analyte concentrator/microreactor chambers

This mode of ACE is currently used primarily as a way (a) to concentrate samples on-column to enhance concentration sensitivity of analytes present in diluted solutions, or at low concentration in complex matrices such as biological fluids; and/or (b) to perform specific biochemical microreactions utilizing minute amounts of materials.

4.1. On-line solid-phase extraction or preconcentration chambers for capillary electrophoresis

One of the main attractive features of CE is the low consumption of sample and reagents. However, this advantageous feature also leads to a severe limitation, namely, poor concentration sensitivity. Often, high analyte concentrations are required to have sufficient material for detection in the capillary. In this regard, a series of solid-phase microextraction devices that are broadly termed ‘analyte concentrators’ have been developed for selective and/or non-selective preconcentration on-line with the CE capillary (for review, see [4,52,114]). Several strategies have been reported for positioning a small section of packing material (impregnated membranes, coated beads, coated polymeric rods, or coated microchannels) in the electrophoresis capillary to capture sample components onto this material. In a subsequent step, a small volume of eluting

solution is passed through the packing to remove the adsorbed material from the 'enrichment chamber.' The eluting solution can be composed of a small plug of an organic solvent, a buffer with a pH range where binding to the ligand is reduced, a solution with an increase ionic strength, a buffer containing special additives, or a mixture of various chemicals (at different pH values) in the solution. The purpose of the enrichment chamber is to allow a larger sample volume (usually >50 μl) to be introduced into the capillary and then to elute the adsorbed solutes in a smaller volume (usually <50 nl) for the separation by CE of the concentrated targeted analytes [4,52,114].

In the original design, the packing material contained an affinity immunoadsorbent [115,116]. Variation of this design contained a reversed-phase high-performance liquid chromatography (HPLC) resin [117–124]. Currently, many new adsorbent materials are being tested [4]. In this section, we will review the likely impact of the technology of CE and the role of the CE analyte concentrator-microreactor on the determination of selected biomolecules present in simple or complex matrices, such as pharmaceutical drugs and substances present in biological fluids. This technique of on-line solid-phase extraction CE allowed for improvement in UV detection limits on the order of 100- to 5000-fold, with even greater gains in sensitivity attainable with injected sample volumes up to 300 μl [4,52,114]. The enrichment chamber also allowed for the performance of a wide range of biochemical and immunological microreactions [4,125]. Applications utilizing various stationary phases and a wide variety of chemistries for immobilization of ligands have been discussed [4,52,114–124] (also see Figs. 13–19).

The simplest enrichment chamber built to date is made of a portion of a capillary containing an immobilized selector that is used as an affinity ligand to capture a specific analyte [126]. Another portion of the capillary is positioned for the actual electrophoretic separation of the analyte(s) after release from the antibodies. To cite one example, antibodies directed against the herbicide atrazine were immobilized by adsorption using a C-8-modified capillary. A fluoroimmunoassay was developed to quantitate atrazine. Because an antibody recognizes only a small

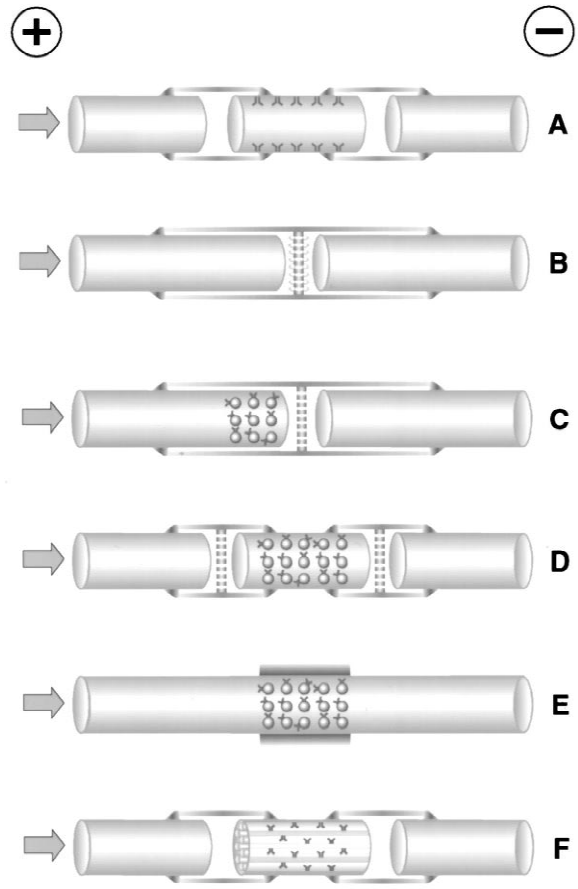

Fig. 13. Schematic diagram representing all six methods currently available to fabricate an analyte concentrator/microreactor chamber, utilizing a variety of different chemistries. Either of the two affinity-related materials (ligand or receptor) can be immobilized directly to a portion of the wall of the capillary, a membrane, a small amount of beads (spherical, irregularly-shaped, magnetic), or a multi-microchannel (multibore) capillary.

part of the antigen (or hapten) molecule, the so-called epitope, these immunoassays are highly specific. Therefore, the results demonstrated a substantial increase in concentration of analytes when using this approach. Important advantages of this on-line concentrator design for CE are the following: (a) the binding of the antibody to the antigen is uniform, providing identical association and dissociation kinetics; (b) the design has no beads or frits, diminishing the chance of blocking or clogging the

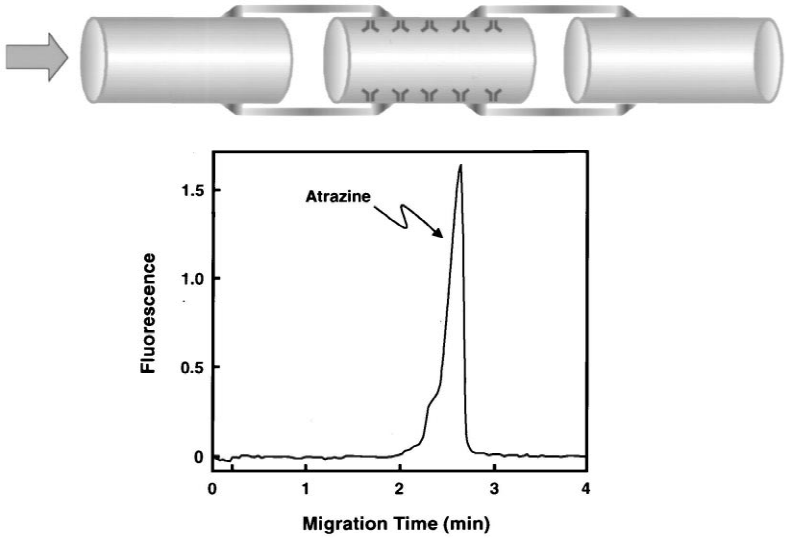

Fig. 14. Schematic representation of an on-line analyte concentrator/microreactor chamber containing immobilized antibodies bound directly to the walls of the capillary. The antibodies were directed against atrazine. This enrichment chamber format contains no beads or frits. One limitation of this scheme is the limited surface area for capturing the antigen. For experimental details, see Ref. [126].

enrichment chamber; and (c) there is a more uniform flow-rate, allowing for more reproducible results. However, there are some limitations in using this approach, resulting in a poor enhancement in the concentration of the analyte, which is due to a restricted affinity surface area of the design. In Fig. 14 the specific binding and separation of atrazine from using antibodies immobilized directly on a

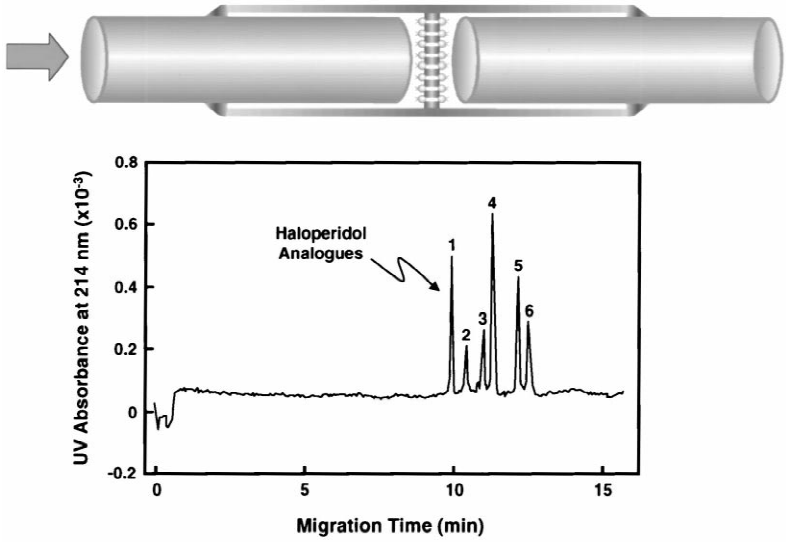

Fig. 15. Schematic representation of an analyte concentrator/microreactor chamber containing membrane-impregnated C-18 material. Several haloperidol analogues were concentrated and separated efficiently using this format. (1) HP^+, (2) HTP, (3) HAL, (4) RHAL, (5) HNO, and (6) HTPNO. For experimental details, see Ref. [52].

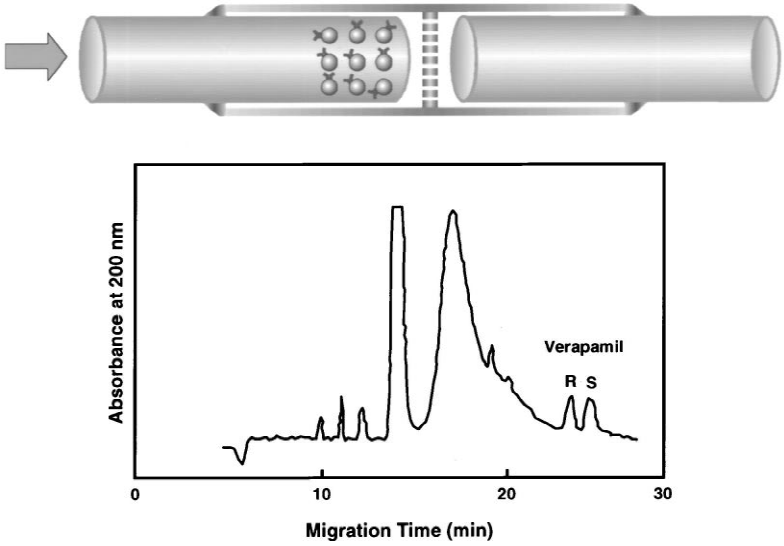

Fig. 16. Schematic representation of a 2-mm analyte concentrator/microreactor chamber containing a HPLC gel C-4 packing material. An aliquot of human plasma ultrafiltrate spiked with 10 μM of a racemic mixture of verapamil was introduced hydrodynamically into the capillary containing the adsorptive packing material. Concentration and separation of the enantiomeric drug verapamil present in a complex biological matrix were accomplished efficiently. For experimental details, see Ref. [123].

portion of the wall of the capillary is shown. Similarly, the development of a surface-bound octadodecylsilane beads-free preconcentrator, for the separation of a mixture of herbicides, was reported [127]. A further use of this beads-free preconcentrator mode, using surface-bound metal chelating

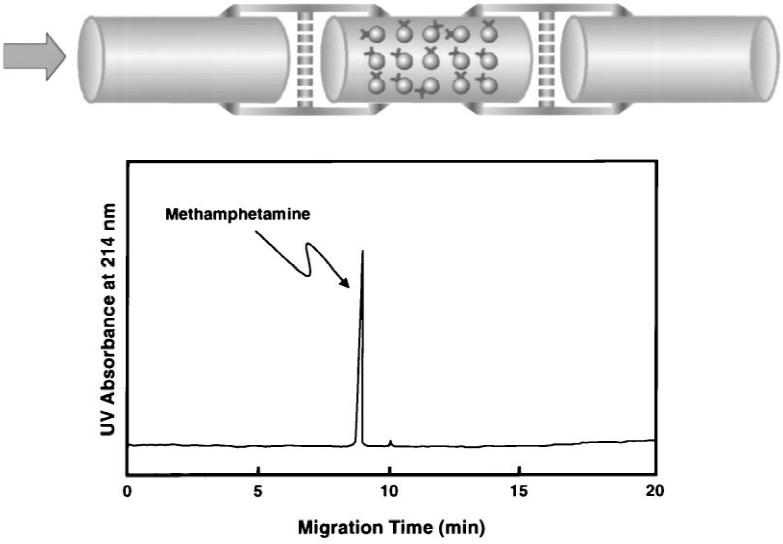

Fig. 17. Schematic representation of an analyte concentrator/microreactor chamber containing immobilized antibodies bound directly to the surface of controlled-porous glass. The antibodies were directed against methamphetamine. For experimental details, see Refs. [4] and [116]. This was the first on-line CE affinity cartridge fabricated for immunotrapping specific antigens found in complex biological matrices.

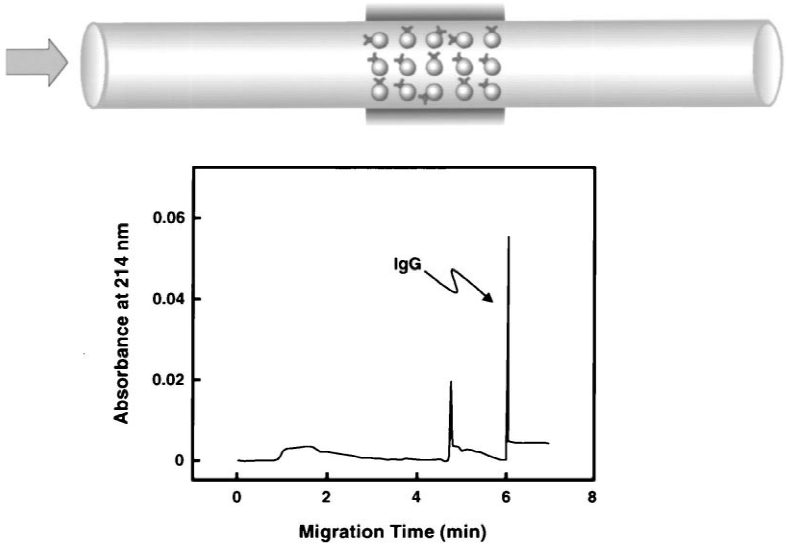

Fig. 18. Schematic representation of an analyte concentrator/microreactor chamber containing beads with immobilized sheep IgG directed against mouse antibodies for the detection of a protein antigen. The antigen in this case was a commercially available mouse monoclonal antibody directed against human growth hormone. Supermagnetic polystyrene microspheres (Dynabeads M-280) of uniform 2.8-μm diameter were used to fabricate the affinity cartridge. The beads were purchased already precoated with sheep anti-mouse IgG. The analyte affinity cartridge consisted of a short plug (2–3 mm) of beads fixed by a magnet placed in the cartridge of the CE system. For experimental details, see Ref. [142].

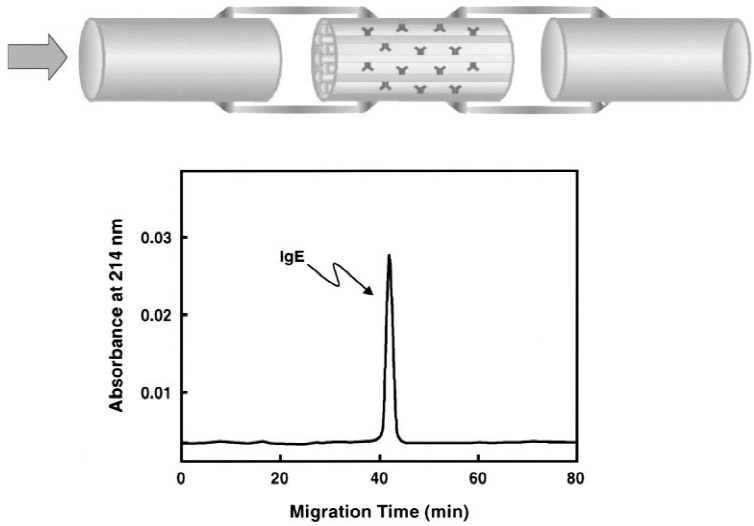

Fig. 19. Schematic representation of an analyte concentrator/microreactor chamber fabricated from a solid rod with a plurality of small-diameter rod passages or through-holes. Each microchannel has sufficient surface area to covalently link monoclonal antibodies directed against IgE to extract IgE molecules present in serum. IgE molecules were bound and eluted from a multi-holes cartridge with a very small amount of high-salt-dissociating buffer containing ethylene glycol. For experimental details, see Ref. [146].

groups, was reported for the separation of proteins that exhibit an affinity for the chelating metal [128].

Another design of on-line preconcentration CE is based on the development of an adsorptive phase made of an appropriate coated/impregnated membrane [4,52,114]. For convenience, this membrane can be installed in a Teflon cartridge system. A typical application for the utilization of this design has been for the investigation of the metabolism of drugs. The metabolic transformation of the neuroleptic drug haloperidol was studied by utilizing a urine sample introduced into a capillary containing a C-18-impregnated membrane. This analyte concentrator enhances sample detectability and desalts the biological fluid that usually has a high salt content. It works primarily with dilute solutions and simple biological matrices, such as urine, but it has some use restrictions when applied to concentrating analytes present in complex matrices. It also has a limited surface area, restricting the concentration capacity of the system. The concentration of samples using this preconcentrator design normally ranges between 100- and 500-fold. In Fig. 15 the binding and separation of haloperidol derivatives using an impregnated membrane as an analyte concentrator are shown. This mode of on-line preconcentration CE also has been used to concentrate and separate peptides found at low concentrations in biological fluids [52,114].

A third on-line preconcentration CE example has been developed utilizing HPLC packing material supported by a single frit, in which both sample introduction and sample separation are carried out in the direction of the electroosmotic flow [123]. The beads are maintained in place by the single frit structure. The feasibility of this sample enrichment technique was demonstrated for the preconcentration and chiral separation of a low concentration of the enantiomers of verapamil, a calcium channel blocker (see Fig. 16). The investigators demonstrated a 200-fold increase in detectability for the determination of unbound concentrations (approximately 5 n*M*) of verapamil enantiomers present in ultrafiltrates of human plasma [123].

The fourth enrichment chamber or preconcentrator cartridge design was essentially the first model ever described in the literature used for preconcentration on-line with CE [115,116]. This design was conceived on the basis of the concept of conventional affinity chromatography reviewed by Cuatrecasas [129] and described elsewhere [130,131]. A high-affinity compound can be attached covalently to an insoluble and porous solid support, which is suitable for chromatography, and utilized to purify a correspondent affinity ligand (present usually in a complex matrix) using column chromatography. In this case [4,115,116], the affinity compound (antibody directed to methamphetamine) was attached covalently to irregularly shaped glass beads (spherical beads made of glass, plastic, or other materials can also be used). The beads were embraced between two frits forming a micro-affinity column located near the inlet of the capillary column. The hybrid column consisted of a 1- to 5-mm cartridge affinity column coupled to a 20 to 60 cm separation capillary. The corresponding affinity ligand, present in a diluted solution or a biological fluid (urine), was introduced into the capillary. Once the affinity interaction between the two compounds was formed, a washing procedure was established to eliminate all salts present in the solution or fluid and to eliminate any other nonrelevant matrix components. The column was equilibrated with an appropriate background electrolyte solution and the bound analyte(s) was eluted in a small plug of an appropriate buffer [4,115,116]. The eluted analyte(s) was then separated by CE (see Fig. 17). This enrichment chamber design permits a larger surface area capacity, enabling a 200- to 5000-fold increase in analyte detectability. Furthermore, when using restricted access media (RAM) [132] for the fabrication of the preconcentrator cartridge [133], it is possible to capture selectively small-molecular mass substances (e.g., terbutaline) enhancing their detectability to at least 5000-fold [133]. Immunoaffinity interactions have been reported that use other separation technologies. Such technologies are primarily on-line immunoaffinity capillary isotachophoresis [134,135] and on-line immunoaffinity chromatography [136–141], allowing determination of analytes in the part-per-trillion range.

The fifth on-line preconcentrator cartridge design was manufactured without the use of frits or membranes. It consists of the introduction into the capillary of a small quantity of magnetic beads containing immobilized biomolecules [142]. The

short plug (2–3 mm) of beads was held fixed by a magnet placed in the cartridge of the CE system. The beads could be replaced for each run, thereby eliminating the need to regenerate the solid support. One of the protocols used for this cartridge design was employed to quantitate an antigen (mouse monoclonal antibody) by using antibodies (sheep IgG directed towards mouse antibody) (see Fig. 18). The assay requires only femtomol quantities of material. Several models of on-line preconcentrator cartidge designs, without the use of frits or membranes, also have been described [143–145].

The sixth on-line preconcentrator cartridge design, also manufactured without the use of frits, beads, or membranes, consists of a bundle of small-diameter capillaries, a solid polymeric or glass rod containing through holes, or a multibore capillary column. The main role of every microcapillary is to serve as a solid support attachment site for chemical substances to which others will be bound [146]. In Fig. 19 an affinity capture of serum immunoglobulin E using immobilized antibodies (in the wall of every single microcapillary) directed against IgE is shown.

Every design mentioned has its advantages and disadvantages. At present, the analyte preconcentrator device is not commercially available, prompting many investigators to create their own modification to the original design [115,116,143,144,147]. Although some difficulties in the fabrication of an optimal CE preconcentrator design have been reported, many investigators have been able to concentrate samples many more fold than the ones reported for other CE methods or devices designed for enhancing sample detectability. Some of these other CE methods include sample stacking, field amplification, and isotachophesis. One example of field amplification has been reported with an accomplishment of a 1000-fold signal enhancement [148]. Other attempts to enhance sample detectability includes the use of CE devices, such as the z-shaped column design or high-sensitivity cell, rectangular capillaries, multireflection cell, axial illumination with axial detection, a single linear photodiode array detector, and the bubble cell [for reviews see [4,52,114,171]]. Clearly, the appropriate solution for a given problem will depend also on the analyte, the matrix, and the separation system. The ideal analyte concentrator/microreactor cartridge should have a large binding capacity (in a small surface area), allowing for a normal electroosmotic flow-rate and the reduction (or elimination) of nonspecific adsorbance of material into the solid support. Although in most cases it will be desirable to have a reusable cartridge, in other cases, it may be necessary to have a replaceable cartridge after each run. More recently, significant improvements have been made in the fabrication of frits. In the original design [115,116,143,144], the frits (located near the injection side of the capillary) were fabricated of porous glass by sintering dry-packed borosilicate microspheres [116–119] or potassium silicate solution [123]. The sintering process was carried out by instant heating of the agglomerated microspheres with a hot nichrome wire or utilizing a laboratory-made microelectric arc device. The purpose of the frits was to form a porous wall, or net structure, to retain chromatographic material into a defined surface area (100–400 μm I.D.×1–5 mm length). Ideally, the porous wall should allow the passage of liquid and solute materials at a normal rate, as observed with other frit materials used in conventional chromatography or HPLC. Several investigators used similar conditions [118–120,147], with the misfortune of not being able to fabricate a uniformly thin frit structure. When monitored under a stereo microscope, the structure appeared bulky and allowed the formation of bubbles. Significantly, after a few uses, they blocked or clogged the system. More recently, other materials have been used that apparently permit a normal electroosmotic flow, reducing the blockage or clogging, and offering a longer use life. Some of these materials include glass fiber [122,149,150], polysulfone membrane filter [124], and Teflon membranes [151,152].

Recently, a uniform multibore capillary column became available commercially (UOP mat/sen, El Dorado Hills, CA, USA). It is made of glass–aluminum or fused-silica glass. This multihole capillary has the potential of being used as a cartridge with a large surface area where chemicals or biomolecules can bind, without the need for using beads, frits, or membranes. It is available as a capillary with a total O.D. of 365 μm that has 600 small bores of 2.7 μm each; this is contained in an internal frit portion with a diameter of 120 μm. Also, is possible to use it as a frit structure [153]. This

homogeneous frit structure should hold beads larger than 3 μm and still maintain a normal flow-rate, diminishing the possibilities of clogging the system. Experiments are now under way in our laboratories to test such multibore capillaries.

Another recent development in on-line preconcentration CE is the use of semipermeable hollow fibers connected to the inlet end of a capillary [172].

In the case of microchips, the volumes of samples are in the femtoliter scale. It may be almost essential to have a preconcentrator/microreactor structure or device in the chip, as the only solution to determine the presence of analytes at low concentrations in biological fluids and other complex matrices.

4.2. On-line microreactors for capillary electrophoresis

Microreactions have progressed rapidly over the last few years. They allow chemical analysis to be carried out in small chambers (microcartridges). They utilize minute amounts of sample and reagent materials while enhancing the speed and information content of analyses. At least, hundreds of potential chemical or biological reactions can be carried out in these microcartridges in a single day. Currently, the ones reported in the literature use immobilized enzymes, antibodies, or lectins. Such immobilized biomolecules enable enzymatic, binding, and/or derivatization reactions to be performed. The most common on-line CE reactions utilized immobilized enzymes that cleave either nucleic acids [154] or proteins [155–162]. As shown in Fig. 20, peptide mapping can be performed in these microreactors on-line with CE. Other studies relate to binding interactions between lectins and sugars [4,163], derivatization of peptides [162], or other analytes [164].

In general, the analyte concentrator–microreactor cartridge is a multipurpose device that has many unique features, such as the following: (a) minimizes sampling handling, (b) concentrates samples present in dilute solutions or samples present at low concentrations in biological fluids, (c) serves as a desalting system and as a cleanup procedure, (d) acts as a chromatographic column allowing the partition of samples for improved separation (even in cartridges of less than 2 mm in length), (e) enables the derivatization of samples to enhance sensitivity, (f) acts as a specific affinity column, (g) supplies a surface as a microreactor chamber to perform a variety of macromolecular interactions including peptide mapping, (h) presents a practical approach to increasing the concentration limits of detection using UV absorbance detectors, and (i) obtains additional information on the analyte when connected on-line with CE–mass spectrometry (CE–MS) [165,173], other detectors such as nuclear magnetic resonance (CE–NMR) [166], or circular dichroism (CE–CD), or a combination of multiple detectors.

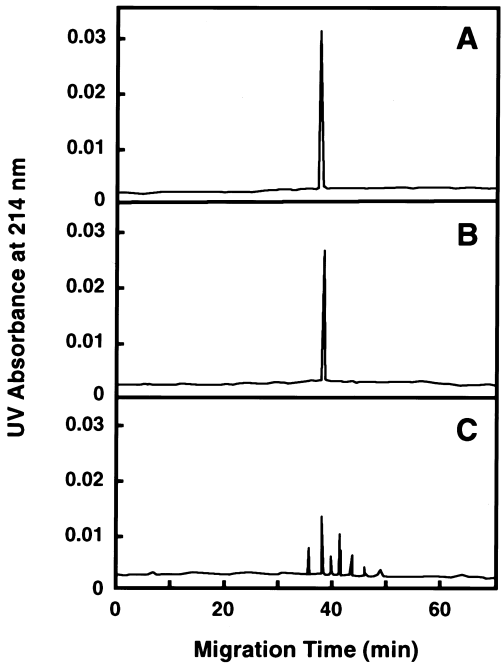

Fig. 20. Schematic representation of an analyte concentrator/microreactor chamber containing an immobilized protease that cleaves efficiently the α-subunit of prolyl 4-hydroxylase [170]. Specificity of protein cleavage was performed by using three different proteins with different biochemical functions. The proteins were bound covalently to irregularly shaped controlled-porous glass under the same experimental conditions, and three microreactors (each containing a separate protein) were fabricated [116]]. Electropherogram of prolyl 4-hydroxylase after the interaction with bead immobilized (A) cytochrome C; (B) bovine serum albumin; or (C) *Staphylococcus aureus* V8 protease. The separation buffer used here was 50 m*M* sodium tetraborate buffer, pH 8.5. For experimental details, see Ref. [162].

5. Future directions of affinity capillary electrophoresis

Affinity capillary electrophoresis has been a useful tool for discoveries in biology and medicine and has promising applications in the field of clinical chemistry. Examples cited in this review, show the feasibility of performing on-line concentration of specific analytes in complex sample matrices using affinity methods. In addition, it presents the possibility of separating efficiently very similar molecules with imprinted polymers present in the capillary. These affinity systems, coupled with the well-known virtues inherent to CE including speed, selectivity, minute sample consumption, automation potential, and flexibility with regard to buffer conditions, may be expected to further the development of its applicability to the determination of biomarkers and drugs and of metabolites of drugs in biological fluids. The expanded structural information available with mass spectrometry and NMR detection systems will increase the applicabilities even further.

Apart from being an analyte enrichment and detection tool, the ACE approach is also, as illustrated above, a means of obtaining a large amount of qualitative and quantitative information on the binding behavior of ions [17], peptides [27,78,167], nucleic acids [30,32], and proteins [25,26,70,79,168,169]. Affinity assays have also been shown to be useful for the quantitative determination of a number of analytes in complex biological matrices, as outlined above.

Limitations of CE-based binding assays appear to be associated mainly with the shortcomings of detectors and the need to optimize separations on a case-by-case basis according to the requirements of the bioaffinity system under study. Even with optimization, it must be realized that some interactions do not have interaction kinetics that will fit into the two main electrophoretic schemes for binding characterization outlined above. The k_{off}-values in the 0.1–0.001 s^{-1} range are especially problematic [21,70]. Also, electrophoresis simply cannot be used to determine binding if the molecular complex cannot be separated electrophoretically from unbound species. Finally, components in proteinaceous samples often participate in capillary wall interactions that may retard or remove analytes and/or influence their binding interactions, and this may make quantitative assessment of binding interactions impossible. However, with capabilities for automation and the development of methods for the processing of more than one sample at a time, in addition to the eventual development of better-designed capillaries and detection systems in the future, immunoassays by CE, especially those based on the competitive setup, appear to hold great promise. On a more fundamental biological level, the wealth of information on the genome and its workings must be translated into functions of biological molecules, which require flexible and precise microtechniques to provide quantitative answers regarding the binding interactions that make all living organisms work. This important field – with the technical improvements mentioned above – will most certainly benefit from the application of CE-based binding assays.

6. Conclusions

Affinity capillary electrophoresis and related affinity microchip technology are important techniques for the study of molecular interactions. They have the potential to revolutionize chemical and biological analysis. In particular, by increasing the speed and information content of analyses, employing automation, miniaturization, and the multiplexed designs, these assays will be applicable to a variety of chemical and biological fields.

7. List of abbreviations

Ab	antibody
ACE	affinity capillary electrophoresis
Ag	antigen
CAGE	capillary affinity gel electrophoresis
CBH	cellobiohydrolase
CE	capillary electrophoresis
CEC	capillary electrochromatography
CD	circular dichroism
DNA	deoxyribonucleic acid
FITC	fluorescein isothiocyanate
Glc	glucose
HAL	haloperidol
HNO	haloperidol *N*-oxide

HP^+	pyridinium species of haloperidol
HTP	haloperidol minus water
HTPNO	haloperidol *N*-oxide minus water
IgA	immunoglobulin A
IgE	immunoglobulin E
IgG	immunoglobulin G
K_d	dissociation constant
k_{off}	dissociation rate constant
L	ligand
LIF	laser-induced fluorescence
Lf	free ligand
M	marker molecule
MIP	molecular imprinting polymer
MS	mass spectrometry
NMR	nuclear magnetic resonance
R	receptor
RAM	restricted-access media
RHAL	reduced haloperidol
UV	ultraviolet

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