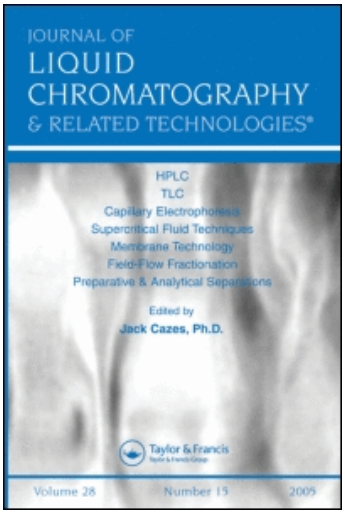

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Microchip Affinity Capillary Electrophoresis: Applications and Recent Advances

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Microchip Affinity Capillary Electrophoresis: Applications and Recent Advances

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Abstract: This review gives the basic principle of affinity capillary electrophoresis (ACE) and examines its utilization in bioscience on microchips. ACE on chip is used as a separation tool as well as for study of molecular interactions. MEKC and chiral separations on microfluidic systems are described. Applications for measuring bioaffinity are focused on enzyme assay and immunoassay that demonstrate a further development of classical ACE in capillaries.

Keywords: Affinity capillary electrophoresis, Chip, Interactions, Electrochromatography, Micellar electrokinetic chromatography, Chiral separation, Enzyme assays, Immunoassay, Biomolecules

INTRODUCTION

Up to now, a variety of capillary electrophoresis based methods for studying interactions have been established. Preferentially, diverse biomolecular interactions are investigated in order to better understand the functioning of living systems. These investigations are crucial for genomic and proteomic research, but also beneficial for other fields of biosciences. Electrophoresis based methods for studying interactions use various experimental approaches and are sometimes summarily termed affinity capillary electrophoresis (ACE). However, no unifying definition of ACE exists in the literature and some

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authors refer ACE to one concrete method for studying interactions. Throughout this review, however, the term ACE is used in its general meaning, which means for all of capillary electrophoresis based methods studying interactions.

An immense number of investigations using ACE methods in capillaries have been published. Since 1995, more than 300 applications for the study of nonspecific and specific interacting equilibrium, including several reviews, have appeared in the literature. On the contrary, only a few articles performing ACE analysis in microfluidic devices, so called microchips or chips, have been published so far.

In ACE, the substances migrating in an electrical field undergo an interaction, which changes their electrophoretical behavior. This effect can be used for the identification and also partly for the quantification of specific binding, or simply for enhancing the separation selectivity. The identification and quantification of an interaction is based on the evaluation of the altered behavior of the substance. Partly, an estimation of the association constant of the interaction is feasible. Affinity interactions, with the objective to enhance separation selectivity, serve mainly for the separation of mixtures of substances with very similar or identical electrophoretical behavior. Typical applications are micellar electrokinetic chromatography (MEKC) and chiral separations.

Since the first description of an electrophoretical separation on a chip (1992), ACE on the chips has mainly been used as a separation tool. Both micellar and chiral separations on microfluidic systems are investigated. On the other hand, only very few applications aimed at the characterization of partition and/or complexation equilibria have been performed on microchips until the present time. One reason for such shortness of publications is the difficulty in electrokinetical control of the sample plug by the simple cross, tee, or double-tee injector, respectively. Another reason is the limitation of detection systems available for planar systems. Most applications deal with fluorescence detection, which has inherently good sensitivity and is, therefore, well suited for the small channel cross-sections. However, most analyte species are not native fluorophores. Such analytes have to be derivatized with a suitable reagent or detected indirectly *via* displacement of a fluorescent ion. Both the derivatization and the addition of a fluorophore to the background buffer may distort the equilibrium in question, as the molecular parameters can be changed. Therefore, fluorescence-labeling-based detection is not well suited for affinity measurements unless the binding is very specific. If the binding occurs between specific regions of the molecules, other parts of these molecules can be labeled without detrimental effects on the interactions. The binding of an enzyme to its substrate, or of an antibody to its antigen, is the typical example of such specific interactions.

For general affinity measurements on microchips, electrochemical detection methods are advantageous due to their easy miniaturization and ability to perform direct detection. UV/VIS absorption is not commonly

used for on-chip detection because the short optical path lengths allow only limited sensitivity. Nevertheless, for analytes available in higher concentrations (0.5-1 mM), UV/VIS detection is still very useful because of its versatility and simplicity.

Both participants of the equilibrium can be injected as sample or rather added to the background buffer. In many cases, the detectability of the compound is decisive for the judgment. Here, we term *S* for solute (usually in the sample) and *L* for ligand (usually in the buffer), whereas *S* and *L* could be all types of molecules as proteins, enzymes, inorganic ions etc.

The review presented here gives an overview of all possible applications of affinity measurements on microfluidic devices published so far. Always, a brief description of the principle of the method including references on review articles is provided. Applications of affinity measurements are divided into two groups according to the goal of the affinity measurement. Affinity measurements serving as a separation tool include chiral separation, MEKC, and chip electrochromatography. Affinity measurements for the characterization of equilibria are comprised of general affinity measurements and special assays, namely enzyme assays and immunoassays.

THEORY

In addition to interactions serving for achieving the separation of complicated sample mixtures and interaction for proper investigation of the equilibrium, some experiments serve only for recognizing an interaction. The result of such experiments is a yes/no answer with regard to the presence of an interaction. Disregarding the purpose of the affinity measurement, three different interaction phases can be distinguished in affinity electrophoresis.[1]

Affinity measurements in free solutions: If the ligand is simply dissolved in the background electrolyte, the interaction takes place in a homogenous solution, which can model the biological conditions inside living organisms. This kind of measurements is, therefore, most widely used for the characterization of the equilibrium. However, certain affinity measurements aimed at the separation of species (such as chiral separations) are also performed in free solutions.

Affinity measurements with pseudostationary phases: The pseudostationary phase is a phase with different physicochemical properties, which is, in contrast to the stationary phase, still in movement with regard to the channel. Interactions taking place in these pseudostationary phases serve mainly for the separation of sample mixtures, but recognition of an interaction (yes/no answer) is also possible. Typical examples of a pseudostationary phase are micelles, dendrimers, or liposomes.

Affinity measurements with stationary phases: Measurements performed in devices containing a solid stationary phase are aimed at separations of mixtures or at the recognition of an interaction. In this concept, features of

both electrophoresis and liquid chromatography are combined and the technique is, therefore, denoted as electrochromatography. The sample components are driven through the device electrokinetically and are separated due to a difference in both electrophoretic mobility (for ionized analytes) and specific interaction with the stationary phase.

Characterization of Equilibria

The characterization of an interaction represents an important tool for a deeper understanding of biological events triggered by specific receptor-ligand interactions. However, the information on an interaction may be useful in many fields of chemistry and other sciences.

In general, interactions are characterized by association constants (frequently also called binding constants) and by the number of ligands that bind to the same class of binding sites present on the solute. For the sake of simplicity, the binding sites within one class are considered identical and the classes are considered independent in the majority of binding studies.[2]

Mathematically, the binding isotherm of such an interaction is commonly expressed by the following equation:

$$r = \frac{[LS]}{c_S} = \sum_{j=1}^{m} n_j \frac{K_j[L]}{1 + K_j[L]} \tag{1}$$

where r is the fraction of bound ligand per receptor or the concentration of ligand bound by one mole of solute; $[LS]$ and $[L]$ are the equilibrium concentrations of bound and free ligand, respectively; c_S is the total (analytical) concentration of the solute; n_j is the number of binding sites of class j, and K_j is the corresponding association constant.

The more classes of binding sites are present on the solute, the more complicated is the calculation of binding parameters. Therefore, the common first approximation is a 1:1 association.[3] The simplified form of Eq. (1) can then be linearized and the respective association constant is thereby calculated. If there is a deviation from linearity observed using this simplification, multiple equilibria have to be considered and non linear models according to Eq. (1) should be used for the calculation of the binding parameters.

Methods for the Characterization of Equilibria

At present there are six affinity electrophoresis modes developed for capillaries, for measuring binding constants. Most of these modes also allow the determination of the number of ligand molecules that bind to the different classes of binding sites.

Methods for the calculation of binding parameters can be divided into three groups according to the way of acquiring binding parameters. The binding parameters can be extracted from the mobility changes, from the peak area of the species, or from the plateau of the elution profile.[2].

Elution profiles of available affinity measurement methods, including the measured parameter of the particular measurement for the calculation of the association constant, are schematically depicted in Figure 1. For explanation see the test below.

Mobility-Shift Assays (Affinity Capillary Electrophoresis)

The mobility-shift assay is sometimes denoted as ACE. Note that this is the narrower meaning of ACE; in this review ACE means any of electrophoretic based methods for studying interactions. Mobility-shift assay is the favorite method in capillary zone electrophoresis (CZE) for the investigation of simple 1:1 equilibria, and it has been recently successfully implemented also for free solution affinity measurements on chips.[4] The separation channel is filled with a buffer containing the ligand in varying concentrations; the solute is injected as a sample. Since the equilibrium is established during the separation, the apparent mobility of the solute depends on the association constant and the mobility difference between ligand and solute. The association constant is thus calculated from the change in the mobility of the solute independent of the concentration of the ligand in the buffer according to Eq. (2).

$$\mu = f([L]) = \frac{\mu_S + K[L]\mu_{SL}}{1 + K[L]} \tag{2}$$

The equilibrium concentration of the free ligand is approximated by the total concentration of the ligand. Obviously, the necessary prerequisite of this method is the difference in the mobilities of the ligand and the solute. This prerequisite is even more demanding if the measurements are realized on a microchip because the separation length is limited to several centimeters at maximum.[4] Small mobility differences cannot manifest in such a short distance. Moreover, short and well defined sample plugs are crucial for high resolution separations. Beside the necessity of small sample plugs in relation to the separation length, a reference compound that does not interact with any type of involved molecule is essential. The mobility change is then referred to the mobility of this compound, a so-called internal standard.

A related method being established only in capillary format so far is the so called vacancy affinity capillary electrophoresis (VACE).[5] In this method, the capillary is filled with a solution, which contains buffer, fixed amount of solute, and varying amount of ligand. A small buffer plug is then injected as a sample and two negative peaks corresponding to the solute and the ligand, respectively, are obtained. The shift in the negative peak mobility of the solute is monitored independent of the concentration of the ligand in the

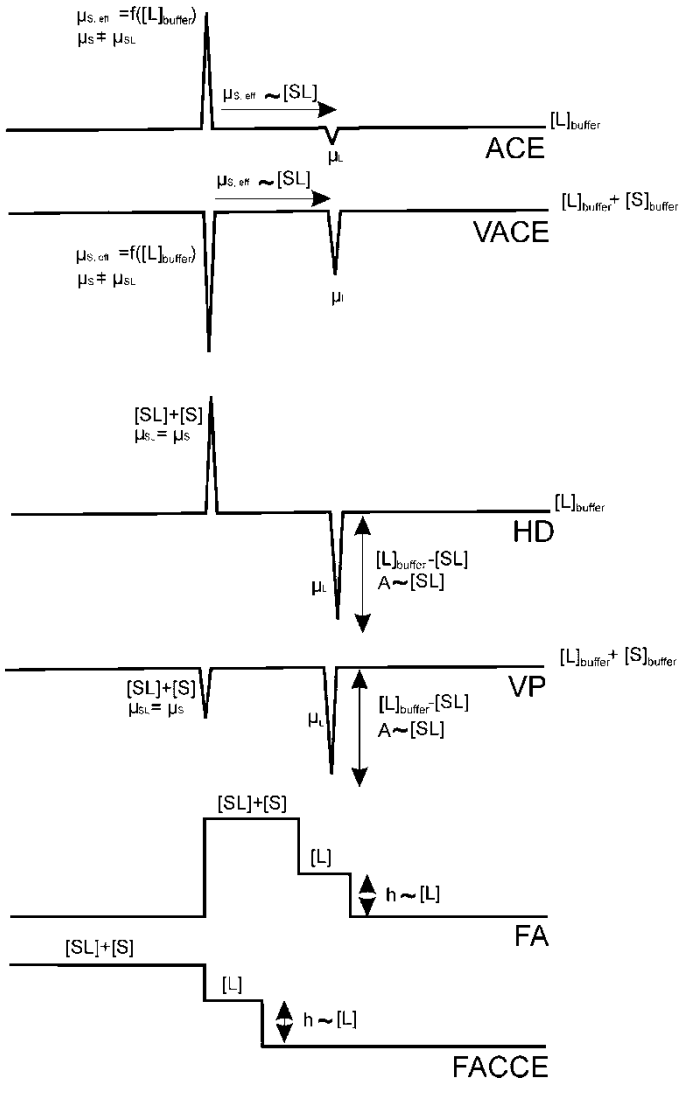

Figure 1. Schematic elution profiles of ACE, VACE, HD, VP, FA and FACCE for the reaction: A – area of detection signal; μ_{eff} – effective mobility. The arrows indicate the parameter $S + L \rightleftharpoons SL$ measured in dependence on the concentration of free ligand, $[L]_{buffer}$, in the background electrolyte.

background electrolyte. This method is advantageous especially in the case of a weakly soluble ligand[3] because the presence of the solute in the background electrolyte helps to solubilize it. VACE is also recommended for interactions of adsorbing species, as the capillary is saturated by all compounds and, therefore, the adsorption is less detrimental. The use of VACE for

adsorbing species and weakly soluble ligands can, therefore, also be suitable for mobility-shift measurements on a chip.

The shift in the mobility of the solute in the absence and presence of the ligand is often used as a proof of interaction, also by affinity measurements with ligands in pseudostationary or stationary phase. The association constant cannot be calculated from these measurements.

Peak-Area-Changes Assays

In free solution methods using peak-area-changes of the solute for the calculation of binding parameters, two different approaches are used depending on the stability of the complex formed by the interaction.

In the case of strong interactions, a direct separation of free and complexed solute is possible and the method is commonly called pre-equilibrium capillary electrophoresis.[6] The solute is pre-equilibrated with different concentrations of the ligand and then injected into the channel filled with buffer. Upon applying high voltage, the free solute is separated from the free ligand and the complex. Peak areas are used for the determination of the equilibrium concentration of free and bound ligand, and the association constant is calculated according to Eq. (1). Calibration measurements are required to relate the concentration to the peak area. This method is applicable to interactions yielding sufficiently stabile complexes with slow dissociation kinetics. Pre-equilibrium electrophoresis on chips is preferentially used in immunoassays where the interaction is strong enough for a direct separation.

In the second approach, the equilibrium is established during the separation, similar to mobility shift assays. Peak-area-changes assays based on this approach are applicable to weaker interactions with fast kinetics and have been established only in capillary eletrophoresis so far. Two methods are available, Hummel-Dreyer method (HD) with the experimental setup identical to mobility-shift assay, and vacancy peak method (VP) with the same setup as in VACE.[7] The peak-area-changes of the ligand, which is added to the background electrolyte in varying concentration, are always evaluated (see Fig. 1).

Generally, the reproducibility of the detection signal is crucial for the ACE methods based on measuring peak-area-changes. In traditional capillary electrophoresis, this is easily accomplished by hydrodynamic injection. On the contrary, microchips rely on electrokinetically driven injections using intersecting channels[8] where the diffusion of the analyte into the separation channel impairs the reproducibility significantly. Several injection strategies such as pinched or gated injections,[9,10] based on applying different voltages during the injection and separation step have been developed to improve the reproducibility, but the voltage control is complicated. However, a pressure driven injection with high reproducibility was recently introduced to microfluidic devices.[11,12] The fast development of affinity

measurements based on peak-area-changes is, therefore, expected in the near future.

Elution-Profile-Changes Assays

Methods evaluating elution-profile-change for the characterization of an equilibrium works with pre-equilibrated samples, which are injected in a large plug (frontal analysis, FA[13]) or continuously (frontal analysis continuous capillary electrophoresis, FACCE[14] to the buffer (see Fig. 1). It is assumed that the mobility of the complex is close to the mobility of the solute and that the mobility of the ligand differs from it sufficiently. Free ligand leaks out from the sample plug because of its different mobility and makes its own plateau. The concentration of free ligand is extracted from the height of the free ligand plateau by means of calibration. Elution-profile-changes assays are employed in capillary electrophoresis so far; the only description of this method on a microchip, however performed in sieving matrix, is given by Backhouse et al.[15] In the publication, DNA (PCR product and primer) was successfully separated in polyacrylimide sieving medium on microchip by using both CZE and FACCE. Even though FACCE was not used for the characterization of any equilibrium there, it clearly shows that frontal analysis with its simple experimental setup could easily be realized on a microchip.

APPLICATIONS OF AFFINITY MEASUREMENTS AS A SEPARATION TOOL

Micellar Electrokinetic Chromatography (MEKC)

MEKC is basically a separation method, which allows a simultaneous analysis of uncharged and charged molecules in untreated capillaries. However, it can be considered as a special case of ACE because it is based on affinity interactions of analytes with micelles. MEKC can easily be used not only for the separation but also for the study of distribution behavior of the analyte. In the latter case, the effect of the micellar composition and the concentration of the surfactant on the alteration of the analyte mobility are investigated. A wide range of applications in pharmaceutics for characterizing partition equilibria between a surfactant and a drug are described. These investigations are focused on the development of effective transport systems for the drugs with respect to their bioavailability. However, all of these studies have been performed in capillaries. Up to the present day, no application dealing with the study of partition equilibria and estimation of partition coefficient can be found for planar microanalytical systems.

MEKC in chip format is not applied very often and uses micelles only for controlling the selectivity and the migration of analytes. The solubilization of the analyte in the micellar phase alters its properties in a significant manner.

Migration times, selectivity, and resolution result from the partitioning of the analytes between micelle and the buffer phase and can strongly be influenced by addition of modifiers. The electrophoretic migration behavior is then determined by the sum of interactions, which the analyte undergoes during the separation process.

Mainly anionic surface-active compounds, in particular sodium dodecyl sulfate (SDS), are used in MEKC.[16–23] SDS and all other anionic surfactants have a net negative charge dependent on the pH value. Therefore, SDS micelles migrate towards the anode, which means in opposite direction to the electroosmotic flow (EOF). Moreover, SDS, similarly as any anionic species, does not interact with the negatively charged surface of the capillary/channels, which is favorable especially in ACE measurements.

Applications of MEKC on a chip are aimed at the improvement of three different goals: separation, partitioning of the analytes, and sensitivity.

Separation

A micellar separation of eight biogenic amines derivatized by fluorescein isothiocyanate (FITC) has been shown with approximately 15 shorter separation times compared to fused silica capillaries.[19] Detection limits satisfactory for analysis in food samples have been reached on a microchip in less than 1 min with the biogenic amines histamine, tyramine, putrescine, and tryptamine.[21] An integrated postcolumn reaction of these amines with *o*-phthaldialdehyde served for their sensitive fluorescent detection. It is demonstrated that SDS stabilizes the EOF in the channels of a cheap plastic chip (made from poly(-dimethylsiloxane)–PDMS) and the separations compare well to glass chips. MEKC separations of explosives on a glass microchip are presented by Wallenborg et al.[22] Also, here SDS micelles are used. Indirect laser-induced-fluorescence served for the detection of the explosives. The resolution enhancement of neurotransmitters by addition of surfactants to the running buffer is demonstrated by Suljak et al.[23] MEKC with amperometric detection has been applied to the separation of these compounds. Here, the sample is continuously introduced into the channel of a microchip with sub-micrometer internal height. Garcia et al.[24] demonstrated the use of anionic surfactants (SDS, sodium deoxycholate, and phosphatidic acid, respectively) in order to increase the EOF, and to enhance the detection signal for a PDMS-microchip. Anionic surfactants adsorbed to the surface of PDMS and affected the zeta potential of the surface and, thereby, the EOF. Also, the electrochemical response for several biomolecules is improved by the presence of anionic surfactants.

Partition Process Adjustment

Microchips are very suitable for precise fluidic mixing and manipulation. Thereby, a solvent gradient for MEKC can be realized in a simple way.

A microchip device, presented by Kutter et al.[16] allows on-chip adjustment of the elution strength of the buffer by the electroosmotic fluid control and by the mobility of micelles. Isocratic and gradient solvent changes on the MEKC separation are controlled by proper setting of voltages applied to the buffer reservoirs of the microchip.

Sensitivity

A sensitivity improvement of lipophilic dyes by on-line enrichment with a sweeping process is demonstrated by Sera et al.[25] The profile of the concentration process and the diffusion during the sweeping was investigated by changing the migration length. Between 90 fold and 1500 fold enhancement in detection sensitivity compared to the normal MEKC mode was achieved for different dyes. The enrichment process is strongly dependent on the partition behavior of analyte and on its migration.

Chiral Separation

Chiral separations represent affinity measurements in free solutions serving for the separation of the enantiomers of an optically active compound. The different interactions of enantiomers present in the sample, in the form of a racemate, with a chiral selector, is the only way to discriminate between them. The chiral selector is dissolved in the buffer and the racemic sample is injected. During the separation, weak complexes between the respective enantiomers and chiral selector are formed and cause the change of the apparent mobilities of the enantiomers. This leads to their separation because the interaction of a chiral selector with each enantiomer is different. The concentration of the chiral selector is varied in order to achieve sufficient separation between the enantiomers. In some applications, the mobility changes of the enantiomers are plotted against the concentration of the chiral selector in the buffer, in order to calculate the association constants of their interaction with the selector.[26] These association constants then serve for the determination of the optimal chiral selector concentration following from the equation:

$$c_{opt} = (K_R K_S)^{-0.5} \tag{3}$$

Enantiomeric separations on micromachined electrophoretic devices are achievable and have been reviewed recently.[27] The applications of chiral separations, performed on a microchip so far (summarized in Table 1), are restricted to the chiral separation of amino acids and biogenic amino compounds. However, the fast development of other chiral separations in micro/nano-channels is expected in the next few years. The main motivation

Table 1. Enantiomer separations on microfluidic devices

Analyte	Derivatization	pH/buffer additives	Detection	Separation length	Year, ref.
Amino acids	Fluorescein isothiocyanate (FITC)	9.2/γ-CD, SDS	Fluorescence	19 cm	1999,[34]
Amino acids	FITC	9.4/γ-CD, SDS	Fluorescence	7 cm	2000,[36]
Amphetamines	Fluoro-nitrobenzofurazane	7.4–8.5/HS-γ-CD, SDS	Fluorescence	14.5–16 cm	2000,[37]
Tryptophan	—	9.1/αCD	Conductivity	9.4 cm	2001,[31]
Neurotransmitter, ephedrine	—	6–12.9/CM-β-CD, HP-β-CD, M-β-CD	Amperometry	8.5 cm	2001,[38]
Amines	FITC	9.0/HP-γ-CD	Fluorescence	7 cm	2002,[39]
Gemifloxacin	—	4.0/crown ether $18C_6H_4$	Fluorescence	10.35 cm	2002,[40]
Amines	FITC	9.2/HP-γ-CD	Fluorescence	8.5 cm	2003,[33]
Neurotransmitter metabolites, precursor	—	2.3–7.2/s-β-CD, CM-β-CD, crown ether, dendrimer	Amperometry	8.5 cm	2003,[32]
Basic and acidic drugs	—	2.5/HS-α, β, γ-CD	UV-detection	2.5 cm	2003,[29]
Amino acids	Fluorescamine	$\leq$9.0/HP-β-CD	Fluorescence	6.2–19.25 cm	2003,[41]
Amino acids	DNS	2.5/HS-γ-CD	Fluorescence	0.7–3.5 cm	2004,[35]
Gemifloxacin	—	4.0/crown ether $18C_6H_4$	Fluorescence	3.8 cm	2004,[28]
Aminoindan	—	7.0/s-β-CD	UV-detection	0.6 cm	2005,[30]

for this development could be fast, qualitative control of pharmaceutical products, or tests of large libraries of enantioselective catalysts.

Similar to all chip applications, fluorescence detection is the most common detection mode in chiral microchip capillary electrophoresis (MCE). An overview of the detection methods used is given in Table 1. In all chiral separations using fluorescence detection, the sample has been labeled outside the microanalytical system. However, due to the flexibility of the chip design, the implementation of on-chip derivatization, as it was shown for MEKC,[21] is expected soon. The potential of chip design versatility was demonstrated by Cho et al.[28] They used a channel-coupled microchip device, which combines a cleaning up of the metal ions present in urine and the separation of gemifloxacin enantiomers by chiral crown ethers. Despite the low sensitivity of UV-detection, several chiral separations applying the commercial instrument MCE 2010 from Shimadzu equipped with UV detector have been shown[4,29,30] For example, Ludwig et al.[29] successfully separated drug enantiomers in 2.5 s. By using highly sulfated-γ-cyclodextrin (HS-γ-CD), low pH and a separation length of 2.5 mm, resolutions up to 12 were reached. One of the few applications using electrochemical detection is demonstrated by Ölvecka et al.[31] In the publication, the enantiomeric separation of tryptophan in the isotachophoresis mode was monitored by conductivity detection. A clear advantage of UV detection and electrochemical methods compared to the fluorescence is the direct measuring without derivatization reactions.

The most widely used chiral selectors in CZE are native and modified α-, β-, or γ-cyclodextrins (CD), chiral crown ethers, proteins, and oligosaccharides. Beside CD, only a few chiral agents have been implemented to enantiomeric chip separations so far. The concurrent use of more interacting agents for the separation of neurotransmitters, their metabolites, and artificial precursors on a chip has been demonstrated by Schwarz et al.[32] Figure 2 compares the effects of two different pairs of interacting agents used concurrently to improve the separation (a and b/c). The sandwich complexes formed by a combination of carboxymethylated-CD (CMCD) and crown ether (Fig. 2(a)) leads to a good enantiomeric separation of adrenalin, but the noradrenalin enantiomers are only partially resolved. Good separation of dopamine, noradrenalin, and adrenalin, albeit not into their isomers, may be achieved by using a dendrimer in the buffer, as shown in Fig. 2(b). The combination of the effects of the dendrimer with the effects of the CMCD, as demonstrated in Fig. 2(c), leads to a complete chiral separation of noradrenalin and adrenalin (dopamine is not chiral) and a good non-chiral separation of methoxytyramine, normetanephrine, and metanephrine.

Besides the selection of a suitable chiral agent, coating of the surface is another way to improve the separation performance in chiral separations. Enatiomeric separations of FITC amino acids have been performed in PVA-coated glass chips.[33] The reduction of analyte wall interactions and reduction in the EOF has led to the clear improvement of the separation efficiency. The

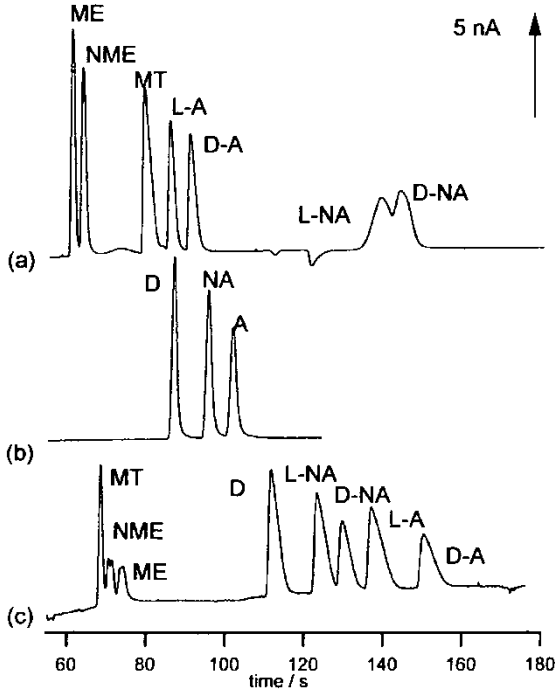

Figure 2. Electropherogram for noradrenaline (NA) and adrenaline (A) in the presence of dopamine (D), methoxytyramine (MT), normetanephrine (NME), and metanephrine (ME). Conditions: buffers, (a) 10 mM TRIS, pH 3.0, 10 mg/mL CMCD, 38 mmol/L 18-crown-6; (b) 20 mM MES, pH 6.0; 5 mg/mL dendrimer; (c) 20 mM MES, pH 6.0, 1.2 mg/mL CMCD, 1 mg/mL dendrimer. HV, 4 kV; detection potential, (a) 1600, (b) 1700, and (c) 1800 mV; injection voltage, 1 kV (2 s); concentration, 100 μM; electrode, Au. Reprinted with permission from [32]. Copyright 2003 American Chemical Society.

separation length of microchip separations undergoes a noticeable trend, as may be demonstrated by the example of enantiomeric separation of amino acids. Whereas Hutt et al.[34] achieved chiral separation of amino acids within 19 cm, Piel et al.[35] chirally separated amino acids derivatized with dansyl chloride (DNS-amino acids) within 0.7 cm, with a separation time of 3.3 s (see Figure 3). Smaller cross section dimensions (50 μm compared to 110 μm) and higher electric field strengths (about 2000 V/cm compared to 520 V/cm) resulted in high resolution separations.

Chip Electrochromatography

Chip electrochromatography is probably the most rapidly growing area of microchip electrophoresis based separations. Specific interactions with

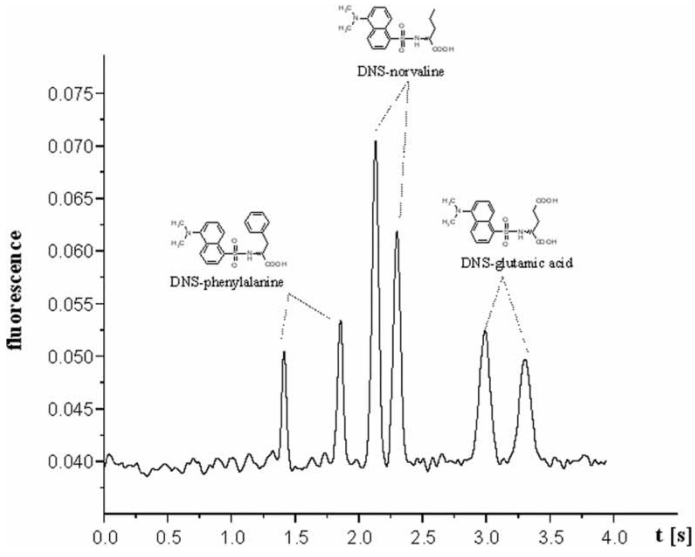

Figure 3. Chiral separation of three compounds in less than 3.5 s. conditions: electrolyte: 2% HS-γ-CD, 25 mM triethylammonium phosphate buffer, pH 2.5. From [35] with permission. Conditions see Table 1.

stationary phases serve for the separation of components. Two main groups of chip electrochromatography, according to the type of stationary phase, are clearly distinguishable: electrochromatography in coated channels (so called open channel electrochromatography) and electrochromatography in filled channels.

Open Channel Chip Electrochromatography

In the open channel electrochromatography, the inner surface of the channel is coated with a stationary phase, which maintains the chromatographic separation mechanism when placed in contact with a mobile phase. In practice, the stationary phase is built via chemical modification of the inner walls. Wall modification on glass microchips has been well established, primarily using silanization, whereas on plastic chips the methods are still under development.[42] Stationary phases for open tubular capillary electrochromatography have been reviewed by Guihen and Glennon.[43]

Filled Channel Chip Electrochromatography

The channel can be packed with particles (beads) or can contain porous monoliths. Packed channels and monoliths have much higher surface area and easily controlled surface chemistry contrary to open channels. Silica

beads are well characterized and easily functionalized; however, uniform and reproducible packing and retaining the beads in miniaturized scale represent a technical challenge.

On the other hand, monolithic stationary phases can be prepared easily and rapidly via free radical polymerization within the channel without need of frits or other retaining structures. Polymer monoliths can posses various functionalities given by the selection of monomers and eventually by their further functionalization. The porosity, the surface area, and the pore size of the monolith are controlled by the composition of the initial monomer solution and by the polymerization conditions. Monoliths, therefore, represent an attractive alternative among stationary phases for microfluidic devices, especially due to its easy preparation in miniaturized formats, and are gaining popularity in the last years.[44] In addition to various porous polymer monoliths, silica based monoliths have been recently introduced as a stationary phase for electrochromatography on microchips.[42] Solid support preparation and their applications on a chip have been recently reviewed by Peterson.[45]

Between open and filled channel stationary phases lie the so called collocated monolith support structures (developed by Regnier and coworkers),[46] a tightly packed array of posts fabricated directly in the channel. These posts divide the channel into a bundle of interconnecting capillaries with frequent mixing nodes, which leads to an increase in surface area compared to open channel stationary phases. Advantages of these support structures are precise dimensions and geometry, and controlled extent of mixing.

Because of the easy preparation of most of the stationary phases in miniaturized formats, a lot of applications for chip electrochromatography either in open tubular or filled channel format can be found in the literature. These applications are not listed in this review because they have been extensively reviewed by Stachowiak et al.[42] in 2004 and most recently by Pumera.[48] For an overview of the applications and a more detailed description of the method, including available technologies, please refer to these reviews dedicated entirely to chip electrochromatography.

In both formats of electrochromatography, albeit in the capillary format, special stationary phases, so called molecularly imprinted polymers (MIPs), have been developed. Molecular imprinting is based on creating a three dimensional cross-linked polymer network containing cavities complementary to the template molecule in terms of size, shape, and chemical functionality. The template is present during the polymerization *in situ* and after its extraction leaves behind a cavity, which is then able to rebind the template (analyte) during separation. MIPs phases are used either in open or in filled channel format, either as a coating, as particles, or as monoliths. MIP phases for capillary electrochromatography have been reviewed by Schweitz et al.[47] MIP stationary phases have not been transferred from the capillary to the chip format to our knowledge so far, probably due to the

short existence of the MIP-technology. Anyway, microfluidic devices based on MIP phases seem to have a promising future.[47]

APPLICATIONS OF AFFINITY MEASUREMENTS FOR THE STUDY OF INTERACTIONS

The most important applications for studying interactions are of biochemical origin. The detailed investigation of chemical reactions and processes inside the cells can provide better understanding of living systems, which is necessary to be able to control and regulate their functioning. In capillary electrophoresis, classical binding studies concerning various drugs, biomolecules, and biomacromolecules are routinely performed and binding parameters are calculated. On the contrary, with microchips, very specific bioassays, such as enzyme assays and immunoassays are most frequently studied. The output of these bioassays is rather different and will be briefly discussed in following sub-chapters. For more detailed insight to enzyme assays and immunoassays performed on microchips, the authors recommend the review from the year 2002 given by Guijt et al.[49]

The only microchip application of classical affinity measurement, including the calculation of binding constants, is a study of the interaction between neurotransmitters as solutes and sulfated β-CD as ligand by Stettler and Schwarz.[4] This paper compares affinity measurements, including calculation of association constants, carried out in microchip and in capillary. The obtained results are shown in Figure 4. Microchip ACE was demonstrated to provide comparable data to the capillary ACE, even though less precise. Affinity measurements on microchips were shown as a low-cost, rapid, and simple screening alternative to capillary.

Enzyme Assays

Enzymes are highly specific catalysts of every reaction inside a living organism. In enzyme assays, the kinetics of the enzymatic reaction is studied and provides information about the affinity of the substrate to the enzyme. The substrate affinity is commonly described by the Michaelis-Menten constant (K_m). However, K_m constant is not equal to the association constant calculated in classical binding studies because it is not measured in the equilibrium state. In case of slow conversion of the substrate-enzyme complex to the final product, the K_m value is numerically equal to the dissociation constant of the enzyme-substrate complex, which is reciprocal to the association constant of the complex. In addition to the kinetics of the enzyme-substrate reaction, the kinetics in presence of different inhibitors is often included in the study and the respective inhibition constants (K_i) are

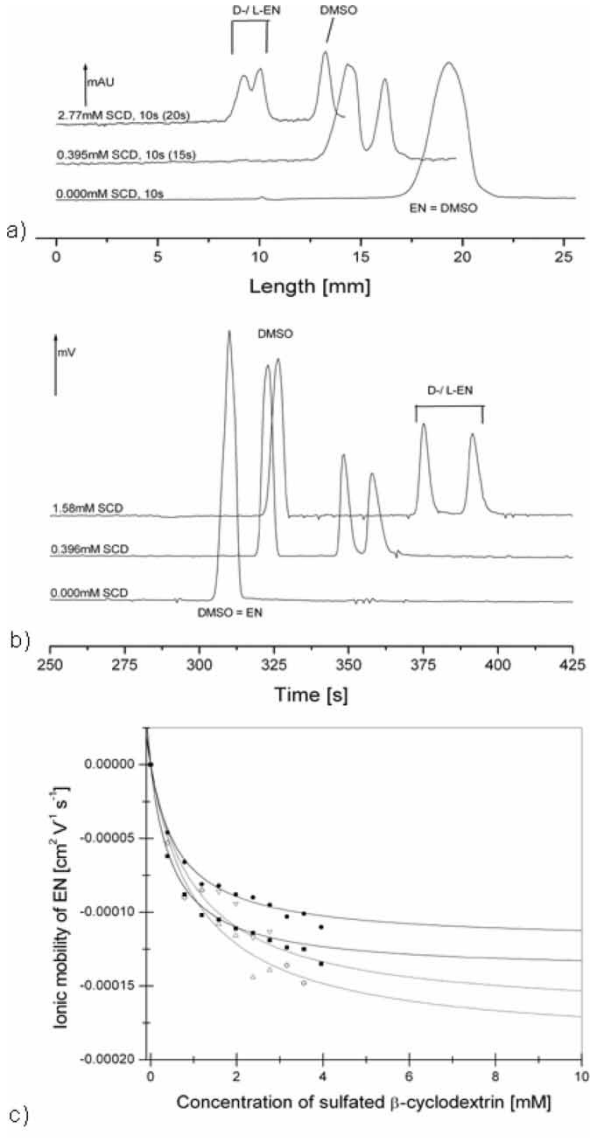

Figure 4. Electropherograms measured on microchip (a) and capillary (b). Buffer: 10 mM TRIS at 210 nm containing sulfated cyclodextrin (sCD); internal standard: DMSO; microchip: 2 mM epinephrine (EN) at pH 7.37. The separation times differed from 10 s up to 20 s (converted to 10 s) at 280 V/cm using an uncoated chip 50 μm × 20 μm i.d. capillary: 0.2 mM EN at pH 7.33. Injection time in each run was 6 s at 25 kV using a 65 cm (79 cm in total) 75 μm i.d. open, uncoated quartz capillary. (c) Affinity capillary electrophoresis: comparison between capillary and microchip. The electropherograms of (a/b) converted into affinity curves with increasing sCD concentrations vs. ionic mobility. Reprinted from [4] with permission from Elsevier. Copyright 2005.

determined. Enzyme kinetics is traditionally studied in cuvettes, where the formation of a product or a consumption of a substrate is monitored.

In the nineties, a new concept in enzyme assay based on capillary electrophoresis has been developed and was denominated as electrophoretically mediated microanalysis (EMMA). The EMMA methodology couples together all of the operations required for the enzyme assay, such as mixing of reagents, initiation of the reaction, incubation of reaction mixture, and detection of the reaction product. Different electrophoretic mobilities of the substrate and the enzyme make it possible to initiate the reaction inside the capillary and to separate the components prior to the detection. A review dedicated to EMMA in capillary, as well as on a microchip, is given by Novakova et al.[50]

Implementation of the microfluidic devices for enzyme assays is even more advantageous compared to the capillary because all of the steps required for the enzyme assays are not only integrated in one device but can also be automatically controlled. A more complex layout of the chip enables the simultaneous screening of several inhibitors in one study.

Enzyme reactions in microscale format are also employed for the determination of compounds. The enzyme either converts undetectable substrate to detectable product or amplifies the detection signal. Such measurements, however, do not belong to the affinity measurements and are, therefore, not discussed in this review.

Generally, enzymes used for enzyme assay can be present in a free solution or immobilized, either over the whole chip or in a restricted area. The overview of enzyme assays performed on microchips so far is summarized in Table 2. Almost all of the measurements used fluorescence detection.

The possibilities, how to design a chip having channels adequately arranged for specific applications, are almost unlimited. For a comparison of the various chips' layout see Figure 5. For example, Ramsey and co-workers[52] studied the enzymatic conversion of acetylthiocholine to thiocholine with an on-chip derivatization of the product (see Fig. 5, in the middle). A possible inhibition of this reaction was investigated by adding an inhibitor, namely tacrine, to the sample. The results of the chip assays have been compared to traditional enzyme assay in a cuvette with good agreement (see Fig. 6).

In free solution enzyme assays, the enzyme is, in most cases, dissolved in the background electrolyte, partly also together with possible inhibitors. The only example of pre-equilibrium electrophoretic study on a microchip, in which both substrate and enzyme are injected as a sample, is given by Wehmeyer and co-workers.[53] Equal incubation times of the sample prior to the injection were important to achieve comparable results. Regnier and Burke[54,55] invented a microfabricated mixer in the cross of the channels in order to improve the mixing of the reagents. The mixing is achieved by transporting the reagents through the mixer consisting of multiple intersecting channels of varying length and width. Microchips have also been used for

Table 2. Chip based enzyme assays

	Solute	Ligand	K	Ref.
In free solution	Resorufin β-D-galactopyranoside	β-Galactosidase and Δc inhibitor	K_m 450 μM, K_i 8 μM	[51]
	Acetylthiocholine	Acetylcholinesterase and Δc inhibitors	K_m 75 μM, K_i 1.5 nM	[52]
	Fluorescein mono-β-D-glucuronide and β-Glucuronidase and Δc inhibitor	—	K_m 18 μM	[53]
	Fluorescein mono-β-D-galactopyranoside and Δc inhibitor	β-Galactosidase	K_m 75 μM	[54, 55]
	Kemptide and ATP[a]	Protein kinase A and Δc competitive inhibitor in a different well	K_m 10 μM, K_i 103 nM, K_m 3.1 μM, K_i 48 nM	[56]
	L-Leucine β-naphthylamine and β-naphthylamine	Leucin aminopeptidase	—	[57]
	Fluorescein diphosphate	Alkaline phosphatase	—	[58]
On stationary phase	*p*-Cresol	Soy bean peroxidase lipase B invertase glucose oxidase	K_m 0.98 mM, K_m 0.59 mM	[59]

[a]Two different chips designs with different methods.

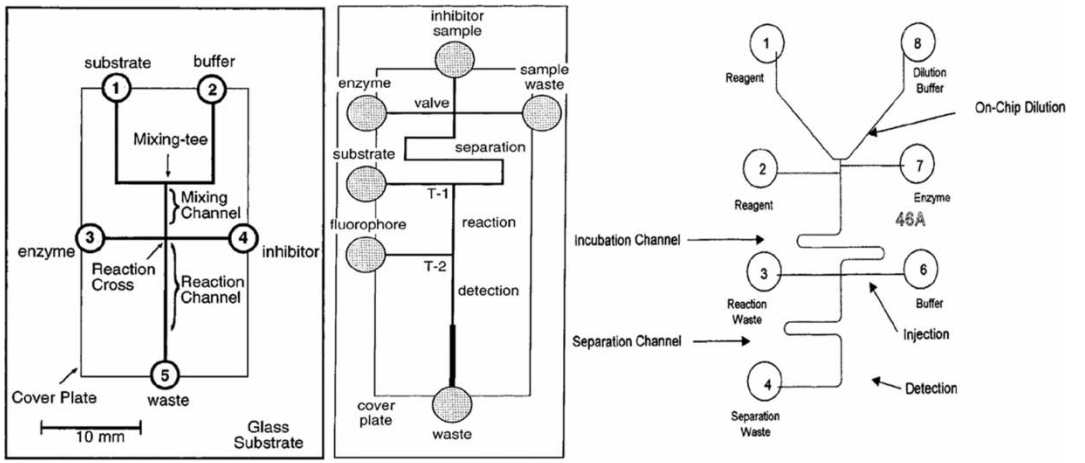

Figure 5. Schematic of the chips used in [51, 52, 56]. With the chip on the left side, dilution of the substrate is possible. Enzyme and a possible inhibitor are added simultaneously. On the chip in the middle, the enzyme first interacts with the inhibitor before coming to the substrate, the product of the enzymatic reaction later binds to the fluorophore and is measured at last. The chip on the right side has two possibilities to dilute the sample before and after the reaction with the enzyme. Reprinted with permission from [51, 52]. Copyrights 1997 and 1999 American Chemical Society. Reprinted from [56] with permission from Elsevier. Copyright 1999.

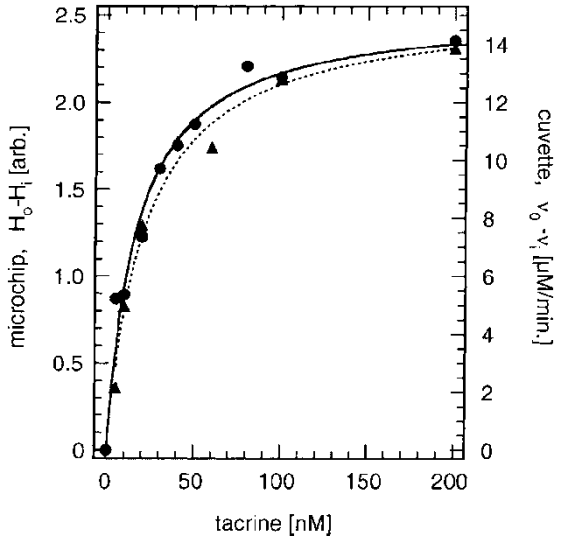

Figure 6. Difference in initial and inhibited enzyme reaction rates versus tacrine concentration for microchip- and cuvette-based assays. Left axis shows values for difference in peak height ($H_0 - H_i$) for the microchip assay (▲); right axis, the difference in initial reaction rates ($v_0 - v_i$) for the cuvette assay (●). Nonlinear least-squares fits are indicated as a dotted line for the microchip assay and as a solid line for the cuvette assay. From (52) with permission.

investigation of more complex enzymatic reactions, which combines more substrates or more enzymes in one assay. Nikiforov and co-workers[56] described a two substrate-enzyme assay for protein kinase A. During the two step reaction the γ-phosphate group was transferred from ATP (first substrate) to a labeled peptide (second substrate). Dordick et al.[59] presented a multienzyme assay with the enzymes immobilized in the channel. The injected substrate is converted stepwise by up to three enzymes and the product of one enzyme reaction served as substrate for the next reaction.

Immunoassays

Immunoassays rely on the affinity reaction between the antigen (a substance recognized by the immune system) and a specific antibody (immunoglobulin (Ig) binding specifically to the antigen). In clinical analysis, the immunoassays serve for identification of the antigen (Ag) or the antibody (Ab) by its selective reaction with the known Ab/Ag. Traditionally, the assay is heterogeneous; the Ag or Ab is immobilized on the surface and the sample containing the other interaction partner is added and incubated there. After removing the unbound fraction of the sample, the bound fraction is washed out and

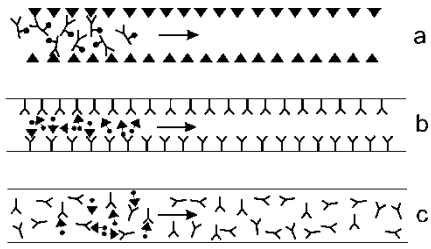

Figure 7. Schema of immunoassays on microfluidic devices.

detected. Labeling based on radioactivity, fluorescence, or on colored enzyme reactions is traditionally used for the detection. In homogenous immunoassays, a separation of the immuno complex from the unbound fraction of the sample is necessary for the evaluation of the assay. High performance liquid chromatography (HPLC), CZE, or newly MCE are used as separation tools. CZE has been also used for the characterization of the equilibrium between Ab-Ag, both in pre-equilibrium electrophoresis and mobility shift assay format, depending on the interaction kinetics. However, because of large variability in the stoichiometry of the Ab-Ag complex, the meaningful interpretation of the binding data often remains an obstacle.[60] As the same problem is encountered in microchips, the use of microfluidic devices is often restricted to the separation of the reaction mixture in homogenous assays. In fact, only a few publications dealing with determination of association constants on microchips have been found.[61,62] However, microchips are also employed for heterogeneous assays, then the chip serves as an immobilization matrix and separation between free and bound Ab/Ag is achieved by a washing step. A schematic drawing of immunoassay arrangement on a microchip in heterogeneous (with Ag (a) or Ab (b) immobilized in the channel) and homogenous (c) format is depicted in Figure 7.

Fluorescent labeling followed by laser-induced fluorescence detection or electrochemical detection is mostly applied for immunoassays performed in microfluidic devices. According to the aim of the particular measurement, a direct or competitive assay is selected. In direct assays either Ag or Ab, forming the immuno complex with the sample in question, is labeled. In competitive assays, labeled and unlabeled Ag or Ab competes for a limited number of the corresponding interaction partner. Applications of immunoassays established on microchips are summarized in Table 3.

Most of the immunoassays serve for the determination of a particular Ag/Ab and not for the investigation of interactions. However, in homogeneous assays the association constants could be determined if the changes in concentration of free and bound Ag/Ab are monitored during calibration measurements. The investigation of the equilibrium in the heterogeneous format is

Table 3. Chip based immunoassays

	Solute	Ligand	Details	Ref.
Homogeneous assays	Cortisol and rabbit anti-cortisol	—	Competitive, determination of cortisol, pre-solved	[63]
	BSA and mouse monoclonal anti-BSA	—	Direct, determination of anti-BSA, pre-solved	[64]
	Theophylline and anti-theophylline	—	Competitive, determination of theophylline, pre-solved	[64]
	BSA and anti-BSA	—	Direct, affinity study, pre-solved	[61]
	TNT, 1,3,5-trinitrobenzene, picric acid, 2,4-dinitrotoluene, 1,3-dinitrobenzene, 2,4-dinitrophenol and monoclonal anti-TNT		Competitive, affinity study, pre-solved	[62]
	Sheep erythrocytes	Rabbit anti IgG	Mobility changes assay, no derivatization, cell counting detection (CCD)	[65]
	Estradiol and anti-estradiol	—	Direct	[66]
	Mouse-IgG	Anti-mouse-IgG	Direct, electrochemical	[67]
	Histamine and anti-histamine	—	Competitive, pre-solved, electrochemical	[68]
	Mouse-IgG	Anti-mouse-IgG	Direct, amperometric	[69]
	Triiodo-L-thyronine	Anti-triiodo-L-thyronine	Competitive, affinity study, amperometric	[69]
Heterogeneous assays	Human IgG	Goat anti-human IgG	Competitive	[70, 71]
	Rabbit IgG	Protein A	Competitive, nonequilibrium conditions	[72]
	D-Dimer	Anti D-Dimer	Direct, electrochemical	[73, 74]
	Atrazine	Anti-atrazine	Competitive, chemiluminescence	[75]

possible only if the incubation is not performed during electrophoresis, thus under non-equilibrium conditions.

The determination of the Ag or Ab by an immunoassay is very selective, sensitive, and is often used in clinical measurements. In general, clinical assays have to be simple, fast, and sufficiently sensitive for detecting the substances in human liquids. Some of published microchip immunoassays[63,64] were shown to fulfill the criteria for the clinical use. Similar to all chip applications, various chip arrangements have been developed for homogenous chip immunoassays. For example, Mathies and Bromberg[62] worked with a special folded channel with several detection points. Harrison and co-workers[66,67] developed a multichannel immunoassay analysis system for measuring up to six independent reactions at the same time. A galvano scanner, moving across the channels, was used for the fluorescence detection. A rather special homogenous immunoassay was performed by Ichiki and co-workers.[65] They have investigated an immunoreaction between sheep red blood cells and rabbit anti-IgG on a microchip coated by gelatine to prevent the cell adsorption. For the detection of the cells a CCD camera, based on counting of the cells, was used. Enzyme mediated detection for microchip immunoassays has been demonstrated, too. An indirect electrochemical detection of Ab and Ag-Ab complex is possible by labeling the Ab with alkaline phosphatase enzyme converting the substrate to aminophenol, which is then oxidized at the electrode.[67] A direct detection mechanism using amperometry for investigations of immunological reactions is described by Wang et al.[69] The principle is based on a ferrocen redox labeling of Ab/Ag and can be applied to a variety of clinical and pharmaceutical immunoassays.

A single use chip with electrochemical detector for the determination of d-dimer in a heterogeneous immunoassay has been developed by Girault and co-workers.[73] A complex immobilization strategy for a heterogeneous immunoassay was published by Thormann and co-workers.[70,71] They covered the channel successively with three layers consisting of biotin-conjugated goat anti-human IgG, neutravidin, and biotin-conjugated dextran, respectively. Labeled human IgG was then shown to bind to the first layer.

FUTURE PROSPECTS

The broadening of the range of application areas and the quantity of utilizations is associated with the commercialization of high performance electrophoretical microchip systems. Such systems represent planar electrophoresis chips with narrow channels of nm dimensions with reliable control of sample and buffer flux equipped with a sensitive detection. Small channel dimensions and high electric field strengths are the most important features for high resolution electrophoresis and are, therefore, substantial for a sensitive recording of mobility shifts. On the other hand, powerful detection

systems are indispensable for identifying minor changes in peak area. The main motivation is given by high throughput analysis, coupling various processes to one system such as pre- and post-channel reactions and the possibility to do parallel measurements in an easy way. The miniaturization, as compared to the ACE in capillaries, enables utilization, cost intensive and uncommon samples, and ligands.

Generally, the transfer of known ACE processes to the miniaturized format is becoming more and more common. Since the basic concept of affinity measurement is derived from capillary measurements, the applicability of microchip investigations depends mainly on the efforts in technology developments as mentioned above. In addition to the transfers of known investigation from the capillary to microchip, a lot of novel microchip applications, including the use of so far unknown ligands for better separation efficiencies and new kinds of noncovalent interactions are, therefore, expected in the near future.

ABBREVIATIONS

A	adrenaline
A	area
Ab	antibody
ACE	affinity capillary electrophoresis
Ag	antigen
BSA	bovine serum albumin
CCD	cell counting detection
CD	cyclodextrin
CMCD	carboxymethyl-cyclodextrin
CZE	capillary zone electrophoresis
D	dopamine
DNS	dimethylamino-naphthalensulfonyl-
EMMA	electrophoretically mediated microanalysis
EN	epinephrine
EOF	electro osmotic flow
FA	frontal analysis
FACCE	frontal analysis continuous capillary electrophoresis
FITC	fluorescein isothiocyanate
H	peak height
HD	Hummel-Dreyer
HS	highly sulfated
HP	hydroxypropyl
HPLC	high performance liquid chromatography
Ig	immunoglobulin
K	association constant
K_i	inhibition constant
K_m	Michaelis-Menten constant

L	ligand
μ_{eff}	effective mobility
MCE	microchip capillary electrophoresis
ME	metanephrine
MEKC	micellar electrokinetic chromatography
MIP	molecular imprinted polymer
MT	methoxytyramine
NA	noradrenaline
NME	normetanephrine
PDMS	polydimethylsiloxane
PVA	poly(vinyl-alcohol)
S	solute
s	sulfated
sCD	sulfated cyclodextrin
SDS	sodium dodecyl sulfate
VACE	vacancy affinity capillary electrophoresis
VP	vacancy peak
ν	reaction rate

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