Microchip electrophoresis bioanalytical applications

Inauguraldissertation

zur
Erlangung der Würde eines Doktors der Philosophie
vorgelegt der
Philosophisch-Naturwissenschaftlichen Fakultät
der Universität Basel

von

Markéta Vlčková

aus Brno (Tschechische Republik)

Basel, 2008

Genehmigt von der Philosophischen-Naturwissenschaftlichen Fakultät der Universität Basel auf Antrag von:

Prof. Dr. Wolfgang Meier
PD Dr. rer. nat. Maria A. Schwarz
Prof. Dr. Edwin Constable

Basel, den 22.04.2008

Prof. Dr. Hans-Jakob Wirz
(Dekan)

“The science of today is the technology of tomorrow”.

Edward Teller

Acknowledgements:

Above all I would like to thank to my supervisor, PD Dr. Maria A. Schwarz not only for giving me the opportunity to work on my PhD in her research group but also for her invaluable help and support during the whole studies.

I would also like to thank my past colleagues Dr. Alexandra R. Stettler and Dr. Pavel Kuban for their big efforts to make me feel comfortable in Switzerland.

Further I would like to express my thanks to Prof. Edwin Constable, Prof. Catherine Housecroft and Prof. Peter Hauser for their concerns regarding my research and for the aid they have provided me.

I am also grateful to the Analytical department of Solvias AG (Basel, Switzerland) for providing time, measurement equipment and conditions allowing a fruitful collaboration with Dr. Dora Bolyan, Dr. Franka Kalman, as well as PD Dr. Maria A. Schwarz.

Next I would like to gratefully acknowledge the financial support from Swiss National Science Foundation, grants Nr. 200020-112190 and 200021-13812/1.

Finally, I would like to thank all my colleagues, friends and all other people who contributed in any way to creation of this thesis. Special thanks belong to my partner, Miroslav Melichar for his everlasting support and encouragement.

Abstract

Microchip electrophoresis (MCE) is a novel analytical technique resulting from miniaturization of capillary electrophoresis (CE) to a planar microfabricated separation device. The consequences of the transfer of CE to MCE in terms of benefits and drawbacks have been identified and commented. The strategies developed to overcome the unfavourable features of the chip with respect to the capillary are briefly described. A method for simultaneous separation of catecholamines and their cationic metabolites has been developed on the microchip. The addition of three modifiers was required to resolve all analytes. The sensitivity of on-chip amperometric detection has been improved by employing an enzyme-catalyzed reaction on the amperometric electrode, as well as by using a carbon nanotube-modified electrode. The developed analytical methodology has been successfully applied for a direct on-chip determination of catecholamines and their metabolites in a mouse brain homogenate. The feasibility of performing affinity measurements as well as isoelectric focusing on the microchip has been demonstrated and available applications of these two electrophoretic modes on a chip have been reviewed. A commercial Shimadzu microchip station has for the first time been applied for high-throughput microchip isoelectric focusing of therapeutic proteins and obtained results have been compared to conventional capillary isoelectric focusing.

List of abbreviations:

5-HT	serotonin
A	adrenaline
ACE	affinity capillary electrophoresis
CE	capillary electrophoresis
cIEF	capillary isoelectric focusing
CNT	carbon nanotube
D	dopamine
DMF	N,N'-dimethylformamide
EOF	electroosmotic flow
EPO	erythropoietin
FAD	flavin adenine dinucleotide
G	glucose
GOx	glucose oxidase
HPCE	high performance capillary electrophoresis
HPLC	high performance liquid chromatography
HVA	homovanillic acid
IEF	isoelectric focusing
LIF	laser-induced fluorescence
LOD	limit of detection
MAB	monoclonal antibody
MALDI	matrix-assisted laser desorption ionization
MCE	microchip electrophoresis
MC-ACE	microchip affinity capillary electrophoresis
MC-IEF	microchip isoelectric focusing
ME	metanephrine
MEKC	micellar electrokinetic chromatography
MHPG	4-hydroxy-3-methoxyphenylglycol
MT	methoxytyramine
μ-TAS	micro-total analysis system
MWNT	multi-wall carbon nanotubes
NA	noradrenaline
NAD(H)	nicotinamide dinucleotide (in reduced form)
NME	normetanephrine
PAMAM	polyamidoamine
pI	isoelectric point
SDS	sodium dodecyl sulfate
SWNT	single-wall carbon nanotubes
VMA	vanillylmandelic acid
WCID	whole-column imaging detection

Table of contents:

1. Introduction

Electrophoresis is a general term, which covers a variety of separation techniques based on different migration of ions and/or charged particles under the influence of an electric field. The migration velocity of an ion is determined by the intensity of the electric field and by the electrophoretic mobility, which is a constant characteristic of the ion for a given medium. Since the discovery of electrophoretic process in 1937 by Tiselius, electrophoresis developed into one of the most widely used analytical separation techniques in chemistry.

Together with the progress in the theoretical knowledge and technical development, attention has been paid mainly to capillary versions of electrophoretic methods, which employ narrow bore capillaries to perform high performance electrophoretic separations. Capillary electrophoresis (CE), or more precisely high performance capillary electrophoresis (HPCE), can be operated in numerous separation modes with different separation mechanisms and selectivities. This makes the technique applicable for separations of virtually all analytes, ranging from small inorganic ions to large biopolymers and even whole cells.

Whereas CE has become a well established technique, a lot of effort in the development is currently devoted to the transfer of CE applications to planar microfabricated separation devices, in other words microchips or simply chips. The process of miniaturization of CE to the chip is facilitated by its relatively simple technical arrangement and by the high efficiency of electrophoretic separations. The main objective of these efforts is to develop so-called micro-total analysis systems (μ-TAS), which will integrate different steps of an analytical process into one miniaturized flow system enabling much faster, fully automated analysis. Even though the development of μ-TAS is still in its early stage, the ultrahigh throughput makes this concept highly attractive in various fields of biosciences, especially in genomics and proteomics, as well as in the pharmaceutical applications.

The two main objectives of this thesis are (i) to provide the theoretical background of microchip electrophoresis (MCE) and (ii) to give an overview of the addressed applications of MCE. Within the theoretical part, the first section is devoted to the description of the differences between the configurations of CE vs. MCE. The

second part of the theoretical section is aimed at characterization of the transfer of CE to the planar microfabricated devices in terms of benefits and drawbacks associated with the miniaturization process. At the same time, the current status of the strategies, that have been developed to overcome the unfavorable features of MCE, is presented. The selected MCE applications cover: (i) a development of a MCE method for simultaneous separation of catecholamines and their metabolites; (ii) approaches to enhance the sensitivity of amperometric on-chip detection of these compounds present in biological samples; (iii) an overview of affinity measurements on the microchip; (iv) a characterization of microchip isoelectric focusing, including a brief summary of microchip isoelectric focusing measurements from the literature.

2. Electrophoresis in capillaries vs. microchannels

2.1. Characteristics of high performance capillary electrophoresis (HPCE)

HPCE is an instrumental analytical technique where the electrophoretic separation is performed in narrow bore capillaries with the internal diameter typically between 10-100 μm. The use of capillary has numerous advantages, particularly with respect to the detrimental effects of Joule heating. The high electric resistance of the capillary enables application of high electric fields with only minimal heat generation, which is furthermore efficiently dissipated because of the large surface area-to-volume ratio of the capillary. The use of high electric fields results in short analysis time and high efficiency and resolution. Moreover, a number of separation modes is available in CE to vary the selectivity, which makes the technique applicable to a wide range of analytes.

The instrumentation of HPCE is uncomplicated and is schematically drawn in Fig. 1. Briefly, both ends of a narrow-bore fused silica capillary are immersed into two reservoirs containing a buffer solution that fills also the capillary. The reservoirs also contain electrodes that provide electrical contact between the high voltage power supply and the capillary. The sample is loaded onto the capillary by replacing one of the buffer reservoirs by a sample reservoir and applying external pressure (hydrodynamic injection) or electric field (electrokinetic injection). After the injection the reservoirs are replaced again, the electric field is applied and the separation starts. The detection is usually performed at the opposite end of the capillary. UV/Vis detection is by far the most common detection technique in CE and is made directly through the capillary wall. Other detection techniques include fluorescence, amperometry, conductivity and mass spectrometry. The modern HPCE instruments are fully automated and thereby allow easy operation and precise quantitative analysis.

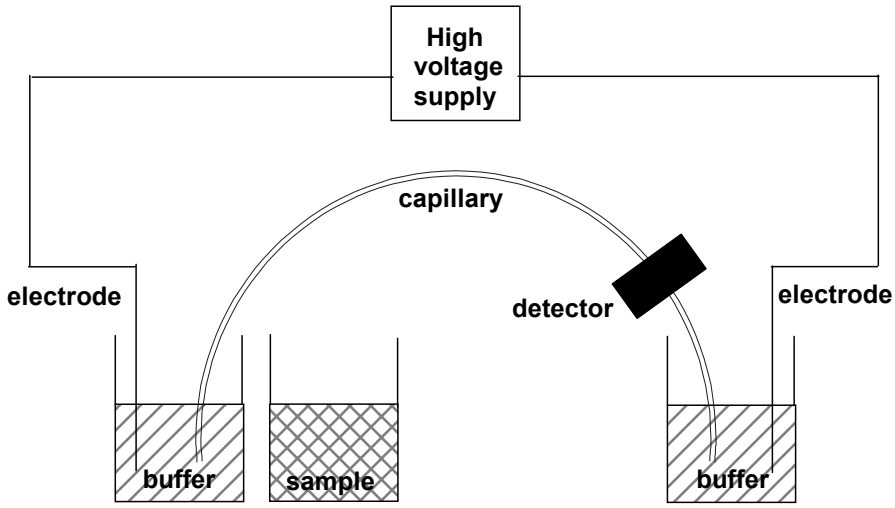


Figure 1. Schematic drawing of an experimental set-up of capillary electrophoresis.

An important feature of CE is the so-called electroosmotic flow (EOF). EOF is the bulk flow of liquid in the capillary, which causes movement of most of the species (regardless of their charge and the hydrodynamic radius) in the same direction and thereby allows a simultaneous separation of cations and anions. The EOF originates from the dissociation of the silanol groups of the capillary wall made from fused silica. The negatively charged capillary wall attracts ions of opposite charge and a solution double layer is formed. The applied electric field causes movement of the cations of the diffuse part of this double layer and attracts them to the cathode. Due to the solvatation of the cations, water molecules are dragged with them and cause the movement of the bulk solution – the EOF. The magnitude of EOF (expressed as EOF mobility) is affected by a number of parameters such as pH of the buffer, its ionic strength, temperature, the intensity of the electric field, and presence of some additives.

Suppression or even reversal of EOF can be achieved through capillary wall modifications, either permanent or dynamic. Permanent modification of the capillary wall is realized by covalently bonded or physically adhered phases. The most common approach for these permanent wall modifications represents silylation followed by deactivation with a suitable functional group (such as polyacrylamide, polyethylene glycol, or polysaccharides). Nowadays, different capillaries with a stable permanent coating are commercially available. On the other hand, a common uncoated capillary is employed in the dynamic modification approach, which is based on addition of an

appropriate modifier to the background electrolyte. This modifier interacts with the capillary wall and in this way affects the EOF. The potential disadvantage of this approach is the sacrifice of the biological-type of conditions, and relatively long equilibration time needed to obtain a reproducible surface.

2.2. Characteristics of microchip electrophoresis (MCE)

MCE results from miniaturization of CE and thus the separation process on the chip is based on the same principle as in the capillary. Both techniques have also a lot of common features, such as high electric resistance enabling application of high electric fields or existence of EOF. Microchip, as well as capillary, offers different separation modes for separation of a wide range of analytes. However, in some aspects the microfluidic separation device has distinctive characteristics resulting from its planar miniaturized format.

The typical microchip design consists of microchannels arranged into a cross shape with one elongated limb (so-called separation channel), where the separation process takes place (Fig. 2). The typical channel depths are 15-50 μm, widths are 50-200μm, and the lengths of the separation channel are 1-10 cm. Four fluid reservoirs are positioned at the ends of the channels; two for introduction of sample and background electrolyte (buffer solution), the other two serving as waste reservoirs. Electrodes present in all reservoirs provide connection to a high voltage power supply.

In contrast to capillary, the injection on the microchip is realized through the intersection of the microchannels, which serves as an integrated injector. The intersection has a form of a simple cross or, alternatively, the form of so-called double tee, where the two arms of the sample channel are offset to form a larger injector region. The sample is usually injected electrokinetically, by applying an electric field across the sample channel. The portion of the sample present in the intersection represents the injection plug, which is subjected to separation when the electric field is applied across the separation channel. The injection and separation steps are often controlled by independent high voltage power supplies.

The detection on the microchip is usually made at the opposite end of the separation channel, most commonly by laser induced fluorescence (LIF) due to its sensitivity. Electrochemical detection methods represent the second most common type of detection on the chip. Recently, mass spectrometry detectors have also been successfully coupled with MCE. In contrast to CE, UV/Vis absorption is not widely used for on-chip detection because of its low detection sensitivity.

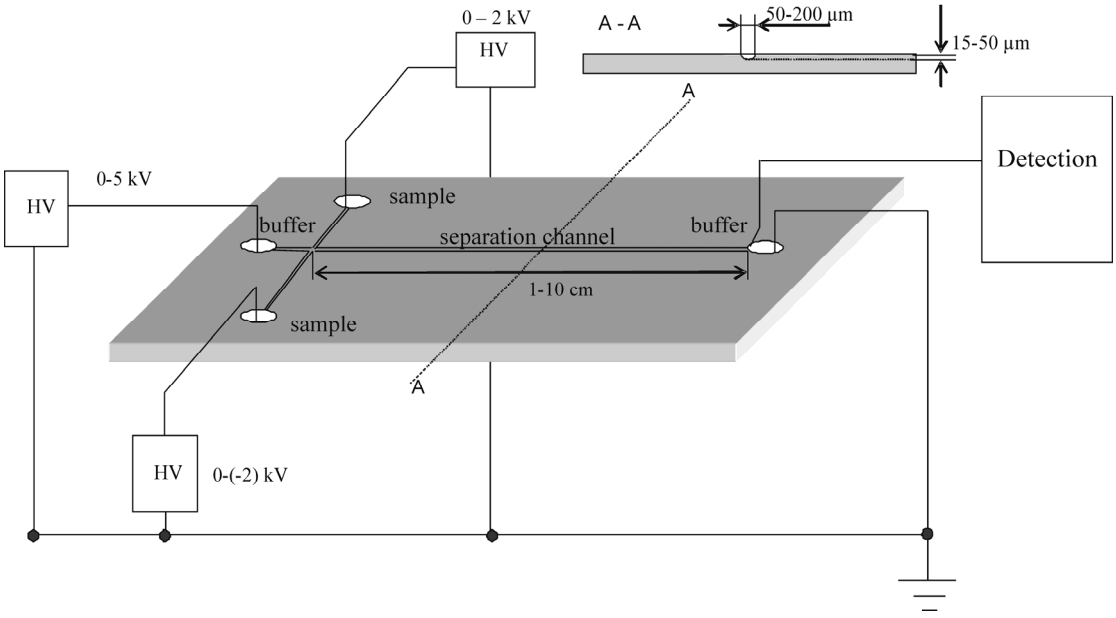


Figure 2. Schematic drawing of the microchip for electrophoresis (HV – high voltage power supply); A-A is cross-section of the device.

3. Transfer of capillary electrophoresis to a microchip

The miniaturization of CE to a microchip is a new promising technology in analytical chemistry, since MCE possesses several advantages above CE. However, the transition from CE to MCE brings along with the benefits also some drawbacks. The overview of advantages and disadvantages associated with miniaturization of electrophoresis is presented in Table 1. The listed items are further described in the following subchapters. In case of drawbacks, a potential solution to overcome the particular limitation is discussed.

Table 1. Overview of the outcomes of miniaturization process

ADVANTAGES	DISADVANTAGES
Reduced analysis time and time for development of a separation method	Limited separation efficiency of zone electrophoretic measurements
Lower consumption of reagents and samples	Imprecise injection
On-line coupling of various processes	Low sensitivity of absorption detection
Potential of parallel measurements	Early stage of commercialization

3.1. Benefits of miniaturization

Obviously, reduced analysis time and lower consumption of reagents and samples are the benefits directly derived from the size reduction of the analytical separation device. The separation on the microchip is completed within a few minutes at most, which leads to a higher sample throughput compared to CE. The rapid analysis also accelerates the development of a separation method because any change in the conditions manifests itself quickly on a chip. Furthermore, the microchip requires only

picoliters of samples and microliters of other reagents and thereby enables utilization of cost-intensive and rare substances.

On-line coupling of various processes to a single device forms the basis of μ-TAS concept, which proposes the integration of the different steps of an analytical process into one miniaturized flow system [1]. Even though the μ-TAS is a new emerging technology and in many ways still in its infancy, the interest in it is growing explosively. A true μ-TAS should contain all of the following elements: a sample preparation component, such as extraction or purification; means to manipulate with the sample, including mixing and reacting with other reagents; a separation step; and a detection component to measure the analytes of interest [2]. Electrophoresis on the microchip proved to be the most practical separation method for the separation step in the μ-TAS concept, since it does not require pumps or moving parts to induce the flow of the fluids [2].

The high sample throughput of the microchip can be further drastically increased using microchip array, as the fabrication of the multiple units, rather than a single unit, does not significantly raise the cost of production. The parallel analyses of tenths or even hundreds of samples have been reported using microchip arrays [3,4].

3.2. Drawbacks of miniaturization

3.2.1. Limited separation efficiency of zone electrophoresis measurements

The separation efficiency can be defined as the ability of a separation system of a given selectivity to resolve the zones of two separated analytes. In zone electrophoresis, the separation is based on differences in mobility of the analytes. The mobility difference necessary to resolve two zones (so-called resolution of the zones) is in zone electrophoresis primarily driven by separation efficiency, which is determined by the zone lengths. The length of the zone is affected by various dispersive effects, which cause the zone broadening (zone dispersion). Under ideal conditions the sole contribution to zone broadening is the diffusion along the migration path (so-called longitudinal diffusion). From this point of view, the short migration paths are favorable

for efficiency, since analytes reach the detector in a shorter time and have therefore less time to diffuse. However, in practice other sources of dispersion are often present and if the contribution of any of them becomes significant, the efficiency and resolution will be sacrificed.

On the microchip, the injection plug length often exceeds the diffusion-controlled zone length and thus becomes the dominant source of dispersion [5]. That is why the separation efficiency of the zone electrophoresis measurements on the microchip is very often much lower than would be theoretically achievable with the short migration paths.

In order to suppress the dispersion contribution caused by the long injection plugs, the width of the injected zone should be minimized. This can be achieved by reducing the dimensions of the cross section of the microchip, which serves as an injector. An example of this strategy is the application of a narrow sample channel having the width five times smaller than the width of separation channel for increasing the separation efficiency [6]. On the other hand, shorter injection plugs require using of more concentrated samples or their effective on-line pre-concentration to achieve the same detection sensitivity.

However, there is a more critical problem associated with the injection, which needs to be solved before the efforts to shorten the injection plugs become truly meaningful. It is the non-reproducible injection procedure resulting from the cross geometry of the injector. This drawback is further discussed in the following section.

3.2.2. Imprecise injection

Electrokinetic injection is by far the most commonly used injection method on the microchips as it does not require an additional mechanic device to move the sample fluid. Using this injection method, the sample is introduced into the intersection by applying the electric field along the sample channel. When the electric field is switched to the separation channel, the portion of the sample present in the intersection is injected and the separation begins. The injection plug length depends principally on the injection time and on the geometry of the cross-section. However, the exact amount of the injected sample cannot be determined since uncontrolled sample leakage occurs during

injection as well as separation step [7]. As the short, well-defined sample plug is crucial for achieving high separation efficiency, various strategies have been developed to improve the injection reproducibility.

3.2.2.1. Improved injection strategies

Several voltage-controlled injection schemes have been proposed to improve the control of electrokinetically injected sample plug. An overview of the main voltage-controlled injection schemes is given in Fig.3.

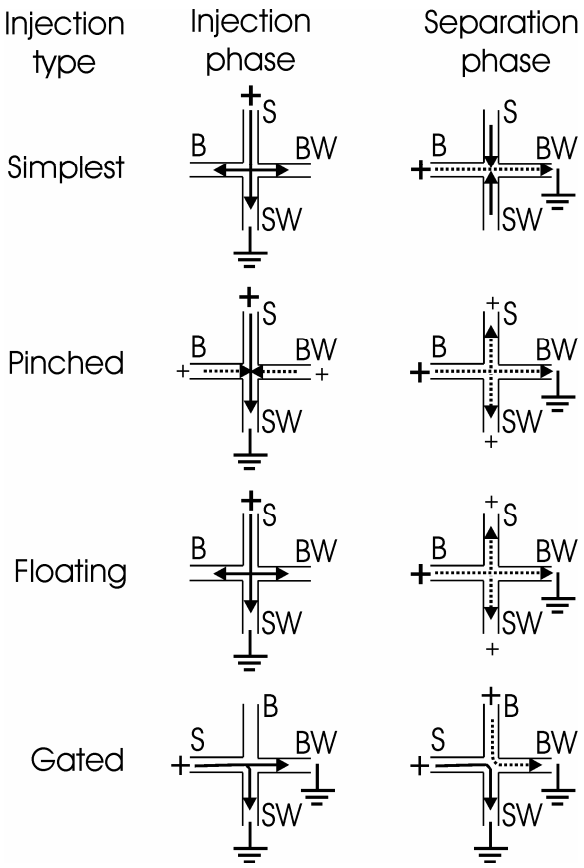


Figure 3. An overview of the basic voltage-controlled injection schemes for electrokinetic injection on the microchip. S – sample, SW – sample waste, B – buffer, BW – buffer waste. The arrows indicate the flow of the sample (solid line) and of the buffer (dotted line) during injection and separation phase.

The most commonly used injection strategy is so-called **pinched injection** [6]. In this injection scheme, pinching voltages are applied at the buffer inlet and outlet during the injection phase. Thereby, a buffer flow toward the sample waste (SW) reservoir is induced to counteract the diffusion of analytes into the separation channel. In the same time, the sample flow is focused enabling an injection of a very narrow sample zone. In the following separation phase, back voltages are applied at the sample inlet (SI) and SW reservoirs to draw the analytes flows back to the reservoirs, preventing sample leakage into the separation channel during the measurement [8]. Even though the pinched injection has a good reproducibility, the exact amount of the injected sample is difficult to determine. In a detailed study [9], it was found out that the injected amount strongly depends on the potentials applied to each reservoir during injection as well as separation phase. Generally, the detection limits of the analytes injected by the pinched injection are much higher compared to the uncontrolled simple injection, as significantly lower amount of the sample is introduced by the pinched injection scheme [2]. On the other hand, the injection of a narrow sample zone has the advantage of enabling a high efficiency on-chip electrophoretic separation.

As a compromise between the injection reproducibility and detection sensitivity, so-called **floating injection** has been proposed [8]. In this approach, the injection phase is carried out without the voltage control and only the subsequent separation phase is controlled by applying the back voltage to the SI and SW reservoirs. The final concentration of the sample, injected using the floating injection, is increased by diffusion of the sample into the separation channel during the injection phase. Obviously, the injection time plays an important role in this injection scheme.

A different approach to the voltage control of the electrokinetic injection represents so-called **gated injection** [10]. In contrast to pinched or floating injection, in the gated injection scheme, the sample flows permanently, making a 90 degree turn at the injection cross toward the SW. Simultaneously, a continuous buffer stream is generated toward the SW and buffer waste (BW) reservoirs, preventing sample leakage into the separation channel. During sample injection phase, the buffer flow is interrupted allowing a plug of the sample to move into the separation channel. The ability of periodical sampling from a continuous flow of the analytes is advantageous

especially for coupling the electrophoresis process with pre-column derivatization [11] or for pre-concentration [12].

However, a general well-known drawback of the electrokinetic injection is the electrophoretic bias in the composition of the injected sample. Discrimination among the analytes occurs during the electrokinetic injection, as the quantity of the injected analytes depends on their electrophoretic mobilites (analytes with higher mobilities will be injected in greater extent than analytes with lower mobilites). The discrimination is prevented in the **pressure-driven sample injection**, which has been recently introduced for MCE [13]. The sample plug is injected either by applying external pressure [14] or using hydrostatic pressure [15]. In addition to it, the injection based on a combination of hydrodynamic (or hydrostatic) and electrokinetic forces has been developed [16,17]. The combined injection is less demanding for the fabrication of the device and substantially reduces the injection bias.

Nevertheless, none of the injection procedures proposed to increase the reproducibility is ideal in terms of a precise control of the injected amount of the sample and total elimination of the sample leakage, as revealed by extensive experimental testing [16] and computer simulations [18]. Consequently, a lot of effort is still put into the technical improvement of the injection for microchips.

3.2.3. Low sensitivity of absorption detection

The **UV/Vis absorption detection** is the most common detection method used with modern chromatographic and electrophoretic separation techniques. This detection method is quite selective and applicable for majority of organic compounds. Moreover, the UV/Vis detector is inexpensive and its implementation is relatively simple. Besides single-wavelength UV/Vis detectors, multiple-wavelength detectors and also diode array detectors are available. Detection of the analytes at several wavelengths is helpful to minimize the absorption of non-relevant peaks. Diode array detectors can be additionally utilized for identification of unknown analytes by comparing the recorded UV/Vis absorption spectrum with spectral libraries.

The sensitivity of the UV/Vis detection depends on molar absorptivity of a given analyte (ε), which is an intrinsic property of that analyte, and on the optical path length. The short optical path length is the principal reason for the low sensitivity of UV/Vis absorption detection on the microchip (due to the small channel depths normally used with the device). The sensitivity of UV/Vis detection on the chip has been enhanced by extending the optical path length by employing Z-shaped cell [19] or using a multi-reflection detection cell with patterned metal mirrors [20]. However, the fabrication of these special detection cells is complicated. Therefore, other detection methods are preferred with the microchip.

3.2.3.1. Other detection methods for microchip electrophoresis

Laser induced fluorescence (LIF) is the most commonly used detection method on-chip because of its high sensitivity. The coherence of the laser beam makes it easy to focus on the small detection cell and to obtain very high irradiation, providing in some applications even single molecule detection limit [21]. Unfortunately, most of the analytes are not native fluorophores and have to be derivatized to be detected by LIF. Moreover, LIF, as well as UV/Vis absorption detection system, is much larger than the microfabricated separation device, which is unfavorable for the development of portable analytical devices.

Electrochemical detection is generally well suited to miniaturized analytical systems and is therefore an attractive detection alternative for MCE, exhibiting a good sensitivity and selectivity. There are several advantages of electrochemical detection over other detection methods, including the ability to miniaturize both the detector and control instrumentation and the fact that many compounds may be detected without derivatization [22]. Four different detection modes can be distinguished in the electrochemical detection: amperometry, voltammetry, conductometry, and potentiometry.

Among these available detection modes, **amperometric detection** remains the most popular electrochemical detection method for MCE due to its easy operation and minimal background-current contributions [22,23]. This detection method is based on

measuring oxidation or reduction currents of the analytes on a working electrode and is therefore restricted to electroactive species [24]. The redox reaction of an electroactive analyte is facilitated by applying constant potential to the working electrode and the generated current is recorded as a function of time. For the coupling of amperometric detection to electrophoretic separation it is important to eliminate the interference resulting from the high separation voltage. Three different approaches have been developed in MCE to isolate the separation electric field. These approaches differ in the location of the working electrode with respect to the separation channel and have been termed: end-channel (off-chip or on-chip), off-channel, and in-channel detection [22]. An overview of the experimental set up of the detection modes for amperometric detection on the microchip is given in Fig. 4.

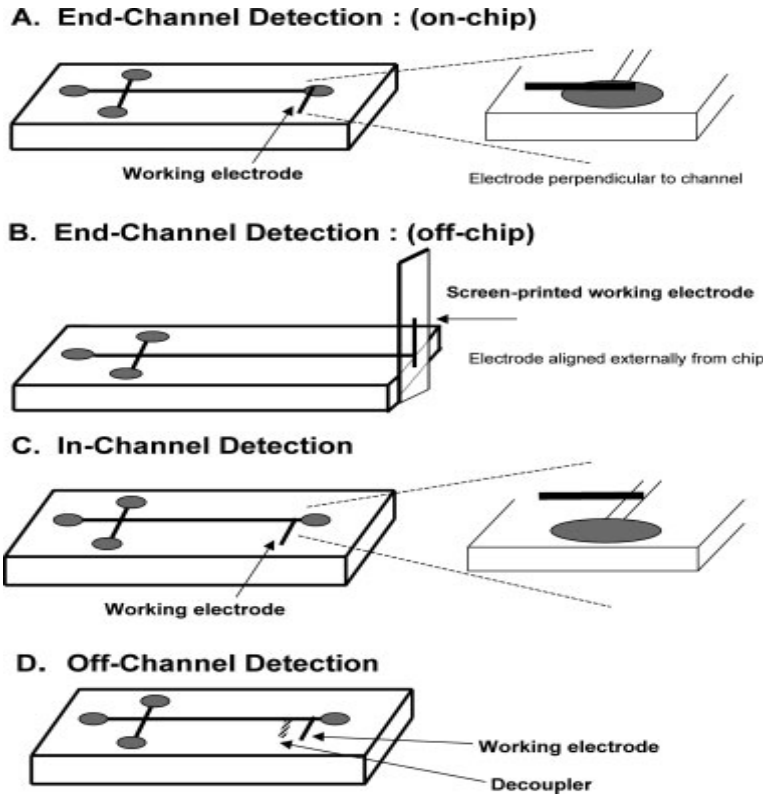


Figure 4. Four available configurations of amperometric detection for MCE that facilitate the isolation of the detector from the separation voltage. From [22].

End-channel detection is the most often used amperometric detection mode for MCE because of its simple experimental set-up [25]. In this configuration, the working electrode is positioned tenths of micrometers from the exit of the separation channel, which allows sufficient decoupling of the separation voltage from the electrode. There are two alignment modes of the end-channel detection, off-chip and on-chip [26]. The former has been prevalent for its convenient replacing and easy cleaning; the latter needs utilization of modern microfabrication techniques to incorporate the electrodes on the microchip. In the on-chip mode of the end-channel detection, the electrode is not exchangeable and this mode is therefore suitable for single-use disposable microchips.

Off-channel detection grounds the separation channel prior to the detection cell by means of decoupler. The decoupler effectively shunts the separation voltage to ground and an electrophoresis-free region is created, where the analytes are pushed past the working electrode by EOF. **In-channel detection** employs an electrically-isolated potentiostat, which enables placing the working electrode directly into the separation channel. Off- and in-channel detection modes eliminate the peak broadening that is characteristic for end-channel detection due to the analyte diffusion occurring in the area between the exit of the separation channel and the electrode.

The conventional potentiostats for amperometric detection need three electrodes for construction of the electrochemical detector cell: working, counter, and reference electrode. Thus, in total four electrodes (three from the potentiostat plus an electrophoretic ground electrode) have to be present at the end of the separation channel in the conventional end-channel detection approach for MCE. However, placing four electrodes at the detector end represents a technical challenge for the miniaturized separation devices, in particular with respect to reference electrode (as it requires internal electrolyte and liquid junction, which is difficult to miniaturize). Therefore, a simplified detection arrangement has been developed, which requires in total only two electrodes – the working electrode and the electrophoretic ground electrode [27]. The latter electrode acts also as a counter to the detector and as a pseudo-reference. A special electronic circuitry is needed for the **simplified two-electrode amperometric detector** [28]. The two-electrode detection arrangement is applicable to a variety of analytes, but the optimum detection potential for each analyte has to be determined experimentally. This is because the detection potential in this arrangement is measured

relative to the pseudo-reference electrophoretic ground electrode, which yields a sufficiently stable potential but its exact value depends on the separation voltage used for the electrophoretic separation [27].

From the other detection methods implemented for MCE, it is important to mention **mass spectrometry**, which is generally a powerful tool in analytical chemistry. The coupling of microfabricated separation devices with mass spectrometric detector is a new emerging technology especially in the area of proteomics. Microchips for MCE have been successfully coupled with mass spectrometer using either electrospray or, newly, matrix-assisted laser desorption ionization (MALDI) interfaces [29]. However, the fabrication of the interfaces is complicated and continues to be a technical challenge. Nevertheless, a lot of progress in this field has been made in recent years and commercialized microchips with mass spectrometric detection for high-throughput proteomics can be expected within the next decade [30].

In all above mentioned detection methods for MCE, the detection is performed at one point of the migration path (located at or close to the outlet end of the separation channel) and the detection signal is plotted against the migration time. In addition to it, **whole-column imaging detection** (WCID) concept has been proposed for specific electrophoretic applications [31,32]. WCID detectors measure absorbance or fluorescence simultaneously along the entire length of the separation channel and in this way may provide detailed insight into the separation process [33]. The separation channel is imaged by the WCID detector often in the real time and the detection signal is plotted against separation length in the course of the separation, thereby allowing concurrently tracing the optimal separation time.

3.2.4. Early stage of commercialization

Commercialization of microscale separation and analysis technology for high throughput applications in genomics, proteomics, drug discovery and medical diagnostics is the main driving force behind the development of the microfabricated separation devices [34]. Even though several commercial systems for MCE analysis have recently become available (for overview see [35]), their application for routine

analyses is still in the test stage. Further improvements in automation and an increase in sample throughput, along with the enhancement of detection sensitivity for analytes present in trace concentrations, can be expected in the near future, which will make MCE technology more widely accepted in commercial laboratories.

4. Selected applications of microchip electrophoresis

4.1. Development of MCE method for simultaneous separation of catecholamines and their cationic metabolites

4.1.1. Overview of the analytes and available analytical methods

The catecholamines dopamine (D), adrenaline (AD), and noradrenaline (NA) are very important neurotransmitters in the mammalian central nervous system and abnormalities in their concentrations and in their metabolism pathways are associated with many neurological diseases such as Parkinson's, Alzheimer disease or Shy-Drager syndrome [36]. D, AD and NA are catabolized to methoxytyramine (MT), metanephrine (ME) and normetanephrine (NME) by catechol-O-methyltransferase; moreover, D and NA are catabolyzed also by monoamine oxidase forming aldehyde intermediate undergoing rapidly further oxidation to an acid or reduction to an alcohol. The major end products of catecholamine metabolism are homovanillic acid (HVA) and 4-hydroxy-3-methoxyphenylglycol (MHPG). The chemical structure of the three catecholamines and of some of their metabolites is given in Fig. 5.

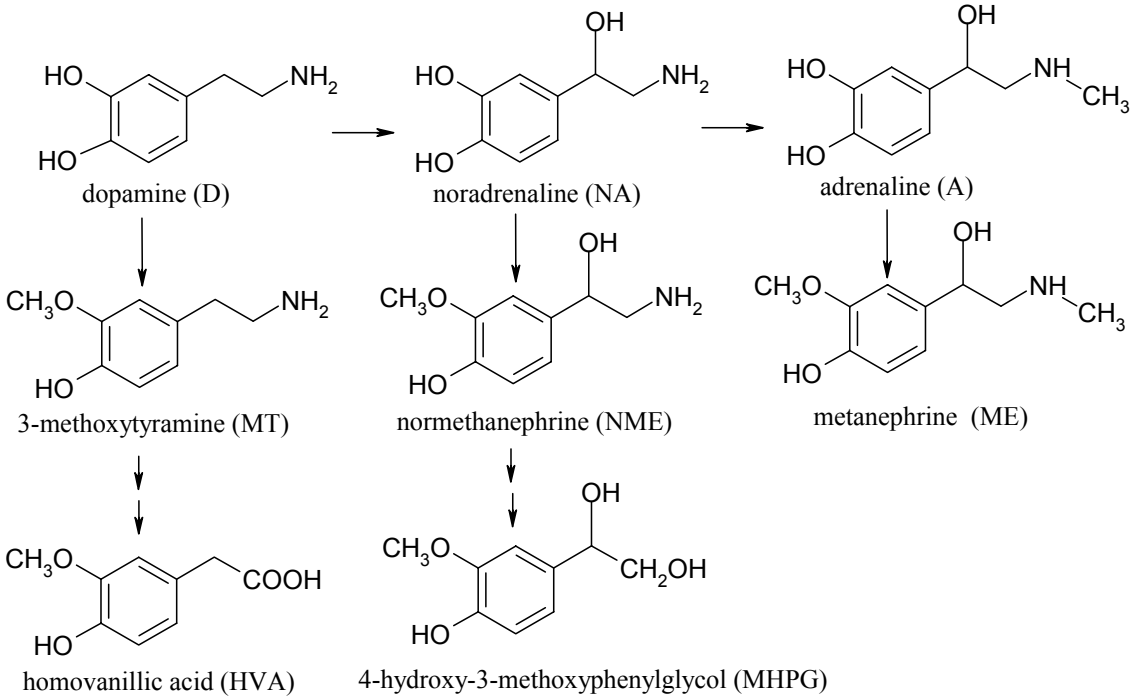


Figure 5. Chemical structures of catecholamines and of some of their metabolites.

Analytical methods are required for the direct simultaneous determination of the catecholamines and/or their metabolites in brain liquid samples, urine as well as blood samples not only for diagnostics of various diseases but also for the investigation of the effect of drugs on the dopaminergic system [37]. Moreover, the measurement of the level of these compounds in the brain homogenates of experimental animals is an important strategy for studying the reactions of the autonomic nervous system [38].

Up to date mainly HPLC (high performance liquid chromatography) methods with electrochemical [39,40] or fluorescence [41,42] detection have been employed for the quantitative determination of catecholamines and their metabolites in clinical samples. Even though chromatographic methods provide low detection limits and good reproducibility, they suffer from high costs, elaborateness and long analysis times.

CE is in general an attractive alternative to HPLC for separation of complex biological samples as it does not require laborious sample clean-up. The direct injection of a biological sample together with a typically faster separation reduces the total analysis time, elaborateness and costs considerably. However, the applicability of CE for simultaneous determination of catecholamines and their metabolites is limited by similar electrophoretic behaviour of these species and by lower detection limits. Despite numerous publications dealing with CE separation of catecholamines and/or their metabolites (for review see [43]), all three catecholamines (D, NA, A) have never been determined along with their cationic metabolites (MT, NME, ME), with solely one exception reported by Peterson et al. [44]. Long, polyvinyl alcohol coated capillary and low pH together with correspondingly long separation time was required for the published separation of all six analytes yet with moderate resolution [44]. Consequently, an effective separation method for fast simultaneous separation of catecholamines and their cationic metabolites for application in clinical laboratories is still being sought.

4.1.2. Application of MCE for fast development of a separation method for selected analytes

One of the attractive application areas of MCE is a cost- and time-saving development of separation methods for complex sample mixtures. Owing to

substantially reduced migration times (about 10-50 times) and to a low consumption of reagents and the sample, new methods, as well as new buffer components, can be effectively tested using MCE. Even though the full separation might not be achievable on the microchip, reasonable predictions on separation selectivity and on the optimal migration times can be made and subsequently transferred to classic CE. Thus, MCE has been applied for fast development of an electrophoretic separation of catecholamines and their *O*-methoxylated (cationic) metabolites. The separation of these species is a demanding task due to their very similar chemical structure (see Fig. 5) leading to nearly identical electrophoretic behavior of these compounds.

The separation of catecholamines and their cationic metabolites on a short migration path (used on a microchip) is impossible without suitable buffer additives, which selectively modify the migration of the analytes. As the conditions of the measurement can be changed promptly on the chip, various modifiers have been tested with the aim to achieve the MCE separation of all selected compounds (for a detailed description see **P1** in the Appendix, section 7.3.). A combination of three additives was necessary to resolve all six analytes; the optimized separation system consisted of 5 mM borate-phosphate buffer (pH 7) containing 10 mM sodium dodecyl sulfate (SDS) and 0.5% (v/v) polyamidoamine (PAMAM) dendrimer of generation 1.5 (Fig. 3 in **P1**). The optimized separation conditions were successfully transferred to CE separation of catecholamines and their cationic metabolites (Fig. 6).

The equilibria taking part during the separation in order to modify the migration of the analytes include: a partition in SDS micelles, complexation of catecholamines with borate, and an interaction with PAMAM dendrimer, which forms a second pseudostationary phase. The overview of the equilibrium reactions is given in Tab. 2.

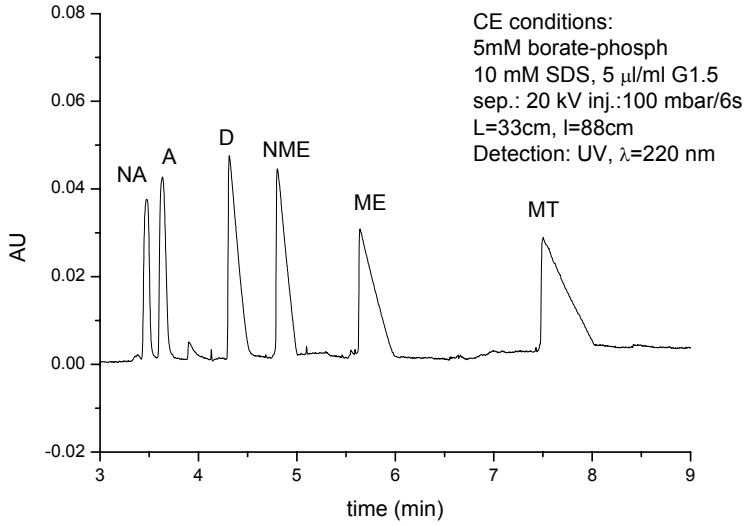


Figure 6. CE separation of catecholamines (NA, A, D) and their *O*-methoxylated metabolites (NME, ME, MT) at the conditions optimized using MCE. Other conditions are given in the Figure.

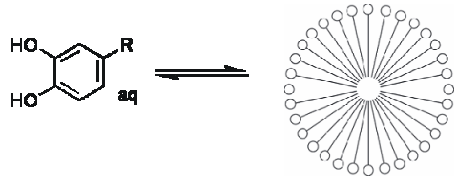
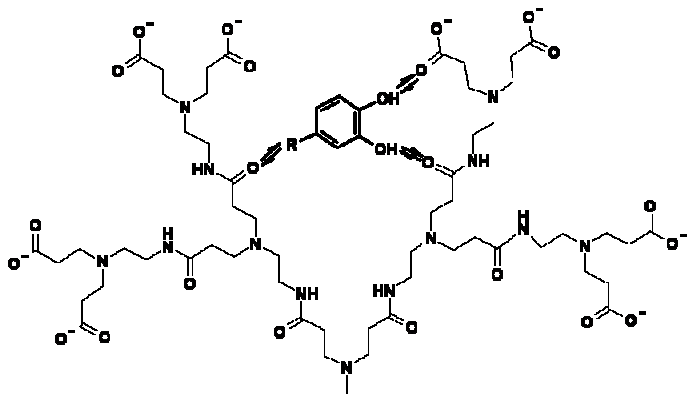
EQUILIBRIUM	DESCRIPTION
	Analytes partition between micellar and aqueous phase; the more hydrophobic analytes, the more retained in the negatively charged SDS micelles.
	Negatively charged complexes are formed between dihydroxy compounds and borate.
	PAMAM dendrimer of generation 1.5 is negatively charged and the strength of its interaction with analytes depends on steric effects (accessibility of the functional groups on the analyte molecule). Only a half of the dendrimer molecule is drawn for clarity.

Table 2. Overview of the equilibrium reactions participating in the electrophoretic separation of catecholamines and their metabolites.

4.2. Enhancement of the sensitivity of on-chip amperometric detection

Another useful application area of MCE is the development of highly selective and sensitive on-chip amperometric detection by means of chemically modified working electrode or using enzyme-catalyzed reactions. Coupling of MCE separation with specifically designed sensitive amperometric detection is an attractive approach to the development of a high throughput analytical device for determination of trace amounts of a selected analyte in clinical samples.

Catecholamines and their metabolites belong to those analytes, which are present in biological samples in very low concentrations. Urine and brain samples contain these compounds in micromolar or submicromolar concentrations; in blood samples is the concentration of catecholamines even in subnanomolar range [36]. Thus, sensitive detection methods are required for the analysis of these species in clinical samples (for review see [43]).

4.2.1. Enzyme-catalyzed reactions

4.2.1.1. Amperometric biosensors

The application of enzyme-catalyzed reactions at the amperometric electrode can amplify the detection signal considerably and thus represents an effective strategy for improving sensitivity of amperometric detection. This concept has been successfully utilized for selective and sensitive measurements of catecholamines and their derivatives in many amperometric biosensors. In such a biosensor, a suitable enzyme is immobilized at the surface of the amperometric electrode and the detection signal is amplified by cycling of the oxidized/reduced analyte between the electrode and the enzyme. Several enzymes (cellobiose dehydrogenase [45], glucose dehydrogenase [46], glucose oxidase (GOx) [47], laccase [48], and tyrosinase [49], respectively) have already been employed in the amperometric biosensors for sensitive detection of catecholamines and related compounds. Two different groups of these enzymes might be distinguished according to the substrate of the enzyme reaction.

First group of the enzymes, so-called reducing enzymes (cellobiose dehydrogenase, glucose dehydrogenase, and GOx), reacts with the oxidation products of catecholamines produced at the working electrode and reduce them back to the original compounds. The original catecholamines are then repeatedly oxidized at the electrode and in this way an amplified oxidation current is recorded by the amperometric detector. The reducing enzymes are then regenerated by a coupled reaction with the natural substrate (cellobiose or glucose), which is added in large excess.

The second group of the enzymes, so-called oxidizing enzymes (laccase and tyrosinase), reacts with catecholamines and oxidizes them to the corresponding *o*-quinones. These are then reduced back at the working electrode, held at a negative potential. The original compounds produced at the electrode are then substrates for the repeated enzyme oxidation. Thus, the reduction current registered by the detector is amplified in these biosensors. The oxidizing enzymes are then regenerated by a coupled reaction with dissolved oxygen.

A further possibility is the use of bienzyme amplification systems, in which both groups of enzymes (reducing and oxidizing) are co-immobilized on the membrane of an oxygen electrode. The cycling of the catecholamine between both enzymes causes the consumption of dissolved oxygen, which is monitored by the detector and is proportional to the concentration of the analyte [46,50].

Except of cellobiose dehydrogenase, all the enzymes employed in these biosensors are incapable of direct electron transfer from the enzyme to the electrode at the working conditions. This is a necessary prerequisite for their successful utilization for sensitive detection of catecholamines and related compounds, because these compounds act as so-called mediators facilitating the electron transfer between the enzyme and the electrode. In the cellobiose dehydrogenase-based sensors a careful adjustment of working conditions is required to suppress the direct electron transfer in favor of mediated electron transfer [45].

4.2.1.2. Sensitive on-chip detection

The concept of enzyme-catalyzed reaction has been successfully adapted for sensitive amperometric detection of catecholamines and their metabolites after their electrophoretic separation on the microchip. By adding GOx enzyme and a large excess of glucose (G) to the background electrolyte, the response of amperometric detector for catecholamines and their metabolites has been amplified by means of cyclic oxidation induced at the working electrode [51]. The limits of detection (LOD) in the range of 10^{-7} M have been reached with the system G/GOx under the optimized conditions.

The active redox component of the GOx enzyme, responsible for cyclic oxidation of catecholamines, is the flavine adenine dinucleotide coenzyme (FAD). Unfortunately, FAD coenzyme is tightly bound to enzymatic protein and incapable of independent existence. The inevitable presence of the protein structure in the separation system however causes lowering of the separation efficiency in time due to the protein adsorption to the walls of the separation channel. In contrast to FAD coenzyme, nicotinamide dinucleotide (NAD) coenzyme can exist independently of the enzymatic protein and as a pure coenzyme might be added to the separation system in a large excess without a detrimental effect on the separation efficiency.

We have demonstrated that the response of amperometric detection of catecholamines and their metabolites can be amplified by adding of the reduced form of NAD coenzyme (NADH) to the background electrolyte (for detailed description see **P2** in the Appendix, section 7.3.). The amplification of the detection signal by NADH is comparable to GOx/G system but NADH-induced cyclic oxidation is applicable at higher detection potentials and hence provides lower absolute LOD. Both amplification systems (G/GOx and NADH) can also be used concurrently and such a combined enzyme system leads to the LOD in the order of 10^{-8} M (Fig. 4 in **P2**). The direct comparison of the detection signals of methoxytyramine (MT) at two different detection voltages (1000 mV and 1200 mV vs. pseudoreference electrode) without any amplification, in presence of NADH, and in presence of combined enzyme system (G/GOx and NADH), respectively, is depicted in Fig. 7.

The amplification principle of the combined enzyme system is schematically given in the inset of Fig. 7. According to our investigations, the two amplification

systems operate independently. The extent of amplification is thus a function of the rate constants of the parallel reactions, of the mobility of the analyte and of the magnitude of EOF. The two latter parameters determine the time spent by the analyte at the detector, which is decisive for the number of redox cycles that can be executed.

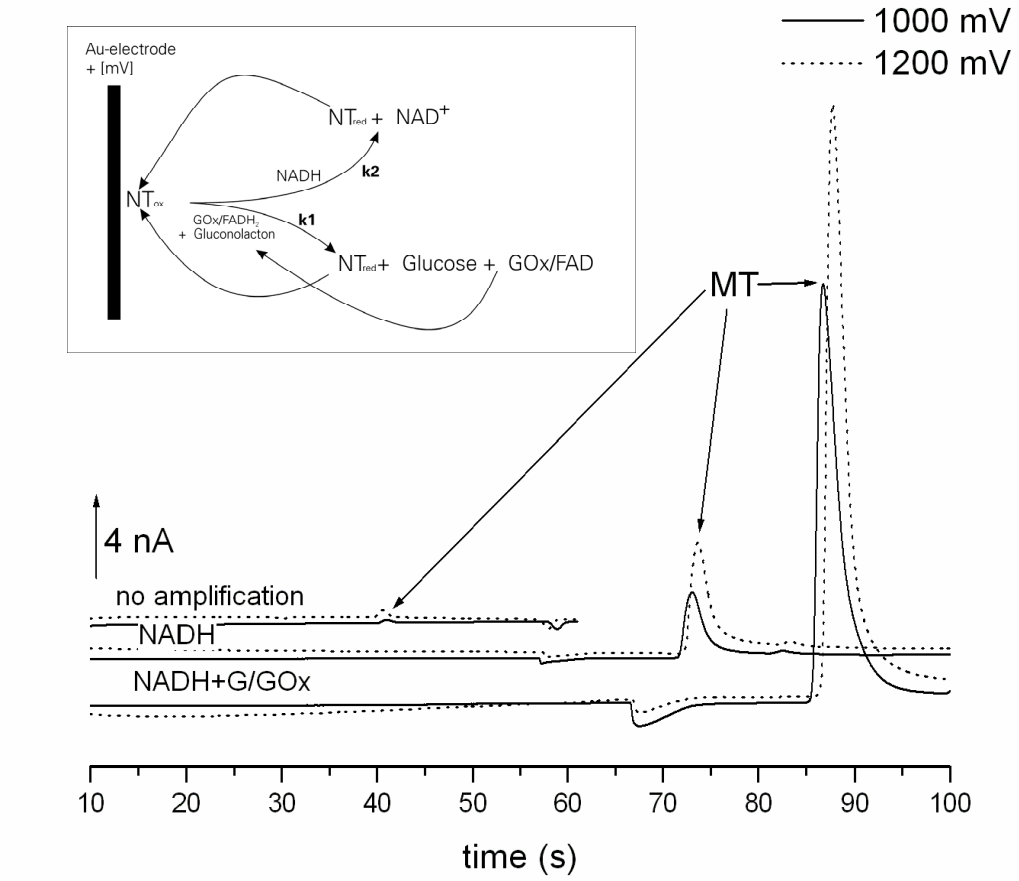


Figure 7. The comparison of the detection signal (at detection potentials 1000 mV and 1200 mV) of methoxytyramine (MT) in 10 mM Tris-phosphate buffer (pH 7): (i) without any amplification, (ii) using NADH amplification (5mM NADH in the buffer) and (iii) using combined enzyme system (5mM NADH, 13 μM GOx and 100mM G in the buffer). Measurements were carried out in constant current mode (1.47 μA). Detection potentials of gold working electrode were set at 1000 mV (solid line) and 1200 mV (dotted line) vs. pseudoreference electrode (simplified two-electrode arrangement was employed). The amplification principle of combined enzyme system is schematically drawn in the inset (NT-neurotransmitter).

4.2.2. Carbon nanotube-modified amperometry

Carbon nanotubes (CNT) are cylindrical carbon molecules with a diameter of a few nanometers and length up to several centimeters. There are two main types of nanotubes: single-walled nanotubes (SWNT) and multi-walled nanotubes (MWNT) (Fig. 8).

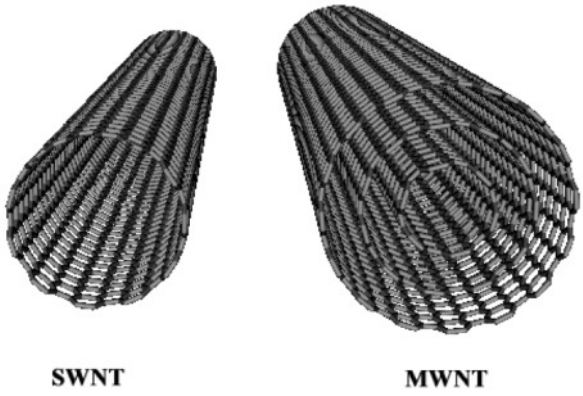


Figure 8. The structures of single-walled carbon nanotubes (SWNT) and multi-walled carbon nanotubes (MWNT).

Both types of CNT exhibit unique geometrical, mechanical, electronic, and chemical properties that make them attractive for a variety of applications. One of the attractive application areas of CNT represents electrochemical detection due to their high surface area, favorable electronic properties and electrocatalytic effects [52]. CNT-modified electrodes were shown to improve electrochemical behavior of various analytes by promoting electron transfer reactions [53]. CNT-modified electrodes have already been successfully implemented for amperometric detection of a variety of analytes including some catecholamines in HPLC [54,55], CE [56], as well as in MCE [57,58].

We have applied a CNT-modified electrode for sensitive detection of catecholamines and their metabolites after their electrophoretic separation on the chip (for details see **P1** in the Appendix, section 7.3.). For the fabrication of CNT-modified electrode, the key step is to obtain a well-distributed and stable suspension of CNT due to their insolubility in most solvents. The following three-step procedure has been adopted for preparation of the CNT-modified electrode. First, purchased MWNT have

been functionalized and purified by refluxing with concentrated nitric acid [53,55]. The functionalization led to the formation of carboxyl moieties on the surface of nanotubes [52]. Second, a stable suspension of CNT has been obtained in *N,N'*-dimethylformamide (DMF) [54,58,59]. Third, the gold working electrode has been covered by DMF suspension by simply immersing the electrode into the suspension followed by drying the electrode in the oven. The last covering step was repeated ten times because it was found out that the amplification of the detection signal, as well as the background noise of the CNT-modified electrode, rose up with the increasing thickness of the CNT layer. Consequently, ten CNT layers was a reasonable compromise between the amplification of the detection response and an acceptable background noise (Fig. 4 in **P1**).

The amplification factors obtained by CNT-modified electrode are generally lower than those obtained by enzyme-catalyzed reactions. However, this concept of sensitivity enhancement is independent of the composition of the background electrolyte and of its pH (in contrast to enzymes that are sensitive to working conditions). Moreover, the CNT-modified electrode preparation is simple and the electrode exhibits a rapid response, catalytic activity, and sufficient stability for multiple runs.

The CNT-modified electrodes are also considered as attractive materials for the immobilization of various biomolecules to create biosensors [60]. GOx has already been successfully immobilized onto CNT by different procedures: physical adsorption [61,62], entrapment in the polymeric matrix [63] or covalent binding [64,65]. However, our attempts to further increase the detection sensitivity for catecholamines and related compounds by immobilizing GOx enzyme on CNT-modified electrode failed. All immobilization procedures (physical adsorption, entrapment in the polymeric matrix, as well as covalent binding) resulted in a direct electron transfer from the enzyme to the electrode via CNT. The constructed biosensor responded directly to the glucose present in the solution and was therefore inapplicable for the sensitive detection of a mediator (in our case catecholamine or its metabolite).

4.2.2.1. Application of CNT-modified electrode for measurement of catecholamines in biological samples

By employing the CNT-modified electrode to enhance the sensitivity of amperometric detection, the method developed for simultaneous separation of catecholamines and their cationic metabolites has been successfully applied for determination of these compounds in biological samples, such as urine and brain samples (for details see **P1** in the Appendix, section 7.3.). The comparison of the MCE separation of standards with direct analysis of the brain homogenate on the microchip is given in Fig. 9. The shift in the migration time of MT can be attributed to the presence of proteins in the brain sample. The peaks detected in the brain sample besides all catecholamines and their cationic metabolites have been ascribed to other metabolites (HVA, and vanillylmandelic acid (VMA)) as well as to another neurotransmitter serotonin (5-HT).

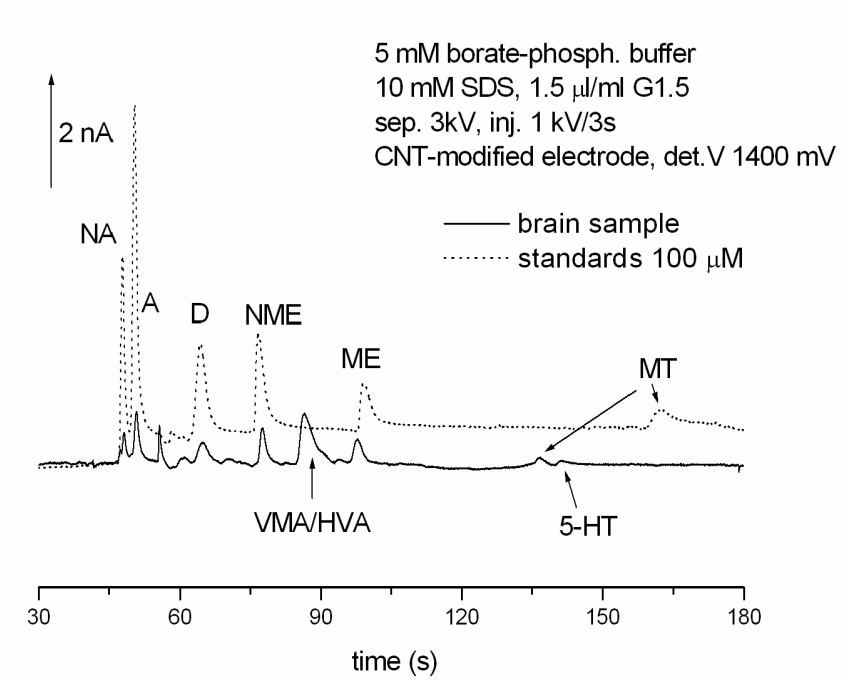


Figure 9. The electropherograms of a standard solution (containing all 3 catecholamines (D,A, NA) and 3 cationic metabolites (MT, ME, NME) at the concentration of 100 μM) and of a mouse brain homogenate (brain sample homogenized in 0.2M $HClO_4$ containing 0.1% (w/v) solution of Na_2EDTA, homogenate was centrifuged, and supernatant was diluted 1:4 with running buffer). Other conditions are given in the Figure.

Unfortunately, the attempt to utilize enzyme-catalyzed reaction for the sensitive determination of catecholamines and metabolites in biological samples was unsuccessful due to the enzyme instability in the complex separation system needed for the simultaneous separation of these compounds.

4.3. Microchip affinity capillary electrophoresis (MC-ACE)

Affinity capillary electrophoresis (ACE) is a term used for CE-based methods for studying interactions. In ACE, a substance migrating in the electric field undergoes an interaction that changes its electrophoretic behavior. This effect can be used for characterization of an interaction or, simply, for enhancing separation selectivity. On the microchip, ACE has been mainly used to enable the separation of mixtures containing substances with very similar to identical electrophoretic mobility.

Typical MC-ACE applications with the objective to enhance separation selectivity are chiral separations and micellar electrokinetic chromatography (MEKC) separations. In chiral separations, a specific compound that interacts differently with the enantiomers of a chiral molecule is employed for the enantiomeric separation. MEKC utilizes micelles, which form a pseudostationary phase, for altering the migration of analytes. Depending on the chemical structure, the electrophoretic behavior of an analyte is more or less affected by its partition between the aqueous and the micellar phase. Mostly, negatively charged SDS micelles are employed for microchip MEKC. An illustrative example of MC-ACE application for separation of a mixture of compounds with nearly identical electrophoretic behavior represents the method developed for simultaneous microchip separation of catecholamines and their cationic metabolites, which employs micelles and other buffer additives to alter the migration of the compounds (see chapter 4.1.).

In the literature, there are only few MC-ACE applications aimed at the characterization of equilibrium presumably due to the imprecise cross injection and limitations of detection systems available for planar systems (see chapter 3.2.). Mostly, specific binding of an enzyme to its substrate and of an antibody to its antigen is

investigated on the chip. The overview of all MC-ACE applications, including the principles of ACE methodology, is provided in **P3** in the Appendix, section 7.3.

4.4. Microchip isoelectric focusing (MC-IEF)

Isoelectric focusing (IEF) is an electrophoretic method developed for separation of amphoteric substances, i.e. substances which can behave as either an acid or a base depending on the surrounding. At certain pH (at so-called isoelectric point, pI), the overall charge of an amphoteric substance, and thus also its electrophoretic mobility, equals zero. This characteristic feature of amphoteric substances serves for their separation by IEF.

The IEF separation takes place in a pH gradient formed along the separation path by special amphoteric buffers (so-called ampholytes). Before starting the IEF measurement, the separation device is filled with a solution of uniform pH, which contains a mixture of ampholytes and a sample. Upon applying the electric field, the negatively charged ampholytes move towards the anode, and the positively charged ones towards the cathode. The ampholytes gradually align themselves between the cathode and anode according to their pI values and form a stable pH gradient. At the same time, the amphoteric sample components are separated and focused to the locations corresponding to their pI values. With the help of synthetic pI markers, the pI values of unknown components may be determined. The experimental set-up of the IEF process, together with the respective pH profile along the separation path before and after the IEF measurement, is schematically depicted in Fig. 10. Proteins and peptides represent the most important analytes for IEF separation. The IEF method is indispensable for identification and characterization of many clinically important proteins, recombinant proteins, cell lysates and other complex protein mixtures.

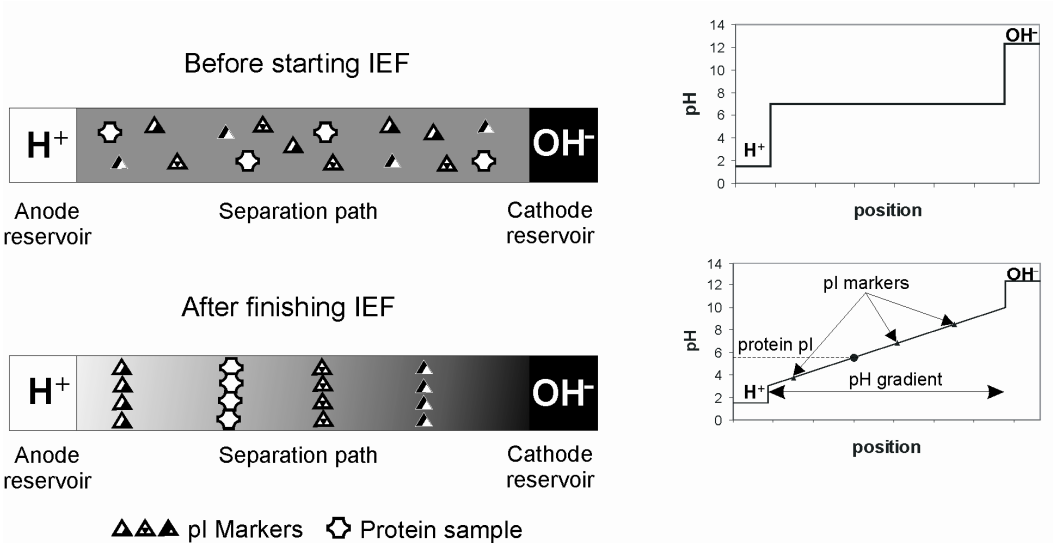


Figure 10. Schematic drawing of the arrangement of IEF separation and the respective pH profiles.

Traditionally, IEF separation is performed in slab gel, which is a time-consuming laborious procedure and provides only approximate results. Capillary IEF (cIEF) offers many advantages over gel-based IEF, such as ease of automation, quantitation, lower consumption of reagents and faster analysis. However, a subsequent mobilization step after finishing IEF process is usually required in cIEF since capillary electrophoresis instruments are mostly equipped with single-point detector. The mobilization prolongs the analysis time substantially and often leads to distortion of pH gradient and thus to poorer reproducibility [66]. MC-IEF results from miniaturization of cIEF to planar microchip format and thus the amount of reagents is further reduced. Also in MC-IEF the mobilization step is necessary if standard single-point detector is employed.

Whole-column imaging detector (WCID) eliminates the need for mobilization and is therefore ideal detector type for cIEF and MC-IEF [67]. A charge-coupled camera of WCID images the entire separation path, and thereby enables a dynamic monitoring of the focusing process. Several kinds of WCID for cIEF as well as MC-IEF have been developed (for review see [33]). Fluorescent imaging detectors are most commonly used WCID because of their sensitivity. UV absorption imaging detectors

are in general less sensitive but can be used for most analytes without labeling reaction. Recently, a commercial instrument for cIEF equipped with UV-WCID (iCE280 Analyzer) has been launched [68]. On the microchip, the realization of WCID is even less demanding compared to capillary due to the planar arrangement and the short migration paths. Consequently, WCID is the prevailing detection mode for MC-IEF applications, even though a specialized instrument for MC-IEF-WCID is not commercially available yet.

In contrast to zone electrophoresis, IEF is an end-point electrophoretic method. Once the proteins get to the positions corresponding to theirs pI values, a steady-state is reached. Because of the focusing effect, sharp protein zones and a high resolution is obtained. Furthermore, the high resolution of IEF is not sacrificed by miniaturizing IEF to the microchip. This characteristic feature makes the MC-IEF applications very promising because the resolution is not compromised by the miniaturization (unlike zone MCE measurements where the miniaturization often leads to lower resolution compared to CE due to limited separation efficiency).

The resolution independency of the separation length in IEF measurements has been proved experimentally as well as theoretically [69-71]. The minimum difference in pI values required for two proteins to be separated ($\Delta(\mathrm{pI})_{\mathrm{min}}$) is expressed by the following equation first reported by Vesterberg and Svensson [72]:

$$\Delta(\mathrm{pI})_{\mathrm{min}} = 3\sqrt{\frac{\mathrm{D}\cdot(\mathrm{d(pH)/dx})}{\mathrm{E}\cdot(-\mathrm{d}\mu/\mathrm{d(pH)})}} \qquad \text{Eq. 1}$$

where D is the diffusion constant of the protein, d(pH)/dx is the pH gradient, E is electric field strength, and dμ/d(pH) is the mobility slope at each pI value. The following assumptions have been used in the derivation of Eq. 1. First, pH gradient is continuous and the applied electric field is constant. Second, both proteins have the same diffusion coefficient. Third, the two adjacent proteins are considered separated when the positions of their peak maximums differ by 3 standard deviations. Forth, there are no effects resulting from EOF, hydrodynamic siphoning, and other such movements. Finally, there is no Joule heating due to high electric currents.

When a constant voltage, V, is applied and a uniform pH gradient is used, then E = V/L and d(pH)/dx = ΔpH/L, where L is separation length. Consequently, Eq. 1 can be simplified to Eq. 2:

$$\Delta(\mathrm{pI})_{\mathrm{mjn}} = 3\sqrt{\frac{\mathrm{D}\cdot\Delta\mathrm{pH}}{\mathrm{V}\cdot(-\mathrm{d}\mu/\mathrm{d}(\mathrm{pH}))}} \qquad \text{Eq. 2}$$

in which, there is no term related to the distance. By shortening the channel length, a higher electric field is applied when the voltage remains the same and the peaks become sharper. The resolution achieved at certain separation voltage is thus maintained even at ultra-short separation paths used on the microchip. The absence of one of the drawbacks associated with the miniaturization of CE to the chip makes MC-IEF probably the most attractive application area of MCE.

4.4.1. Evaluation of MCE-2010 chip station for MC-IEF

Shimadzu microchip electrophoresis system MCE-2010 is a commercial instrument equipped with linear imaging UV photodiode detector, which has been originally developed for high-throughput DNA analysis. The combination of high throughput and the linear imaging detector makes the MCE-2010 chip station useful also for MC-IEF separations. However, the instrument has not been applied to MC-IEF measurements so far. The applications of MCE-2010 from the literature cover chiral separations [73,74], zone electrophoretic analyses [75-77], and affinity measurements [5].

We have demonstrated that MCE-2010 is applicable to MC-IEF separations (for details see **P4** in the Appendix, section 7.3.). However, certain adjustments are required to adapt the MCE-2010 apparatus to MC-IEF measurements. The Shimadzu microchip has been designed for zone electrophoresis measurements (Fig. 11) and thus has some features, which are unfavorable for MC-IEF. The cross injector of the microchip is not well suited for IEF measurements, since the sample present in sample reservoir and sample channel can enter the separation channel during the focusing and thereby disturb the focusing process. Nevertheless, the undesired flow of the sample during focusing could be prevented by applying a pinched voltage to both sample reservoirs (SI and SO)

during the IEF measurement. Furthermore, on the Shimadzu chip, the IEF process takes place in the whole separation channel between the reservoirs BI and BO with the total separation length of 40 mm. However, the linear imaging detector of MCE-2010 apparatus covers only a section of the separation channel of 25 mm, starting from the cross injector onwards (Fig. 11). Consequently, the pH gradient has to be adjusted according to the pI values of the analytes in order to ensure their focusing within the imaged part of the separation channel. Using developed strategies for adjustment of the pH gradient, analytes with pI values from 2.85 to 10.3 could be detected by the imaging detector (see Fig. 3b in **P4**), which is well comparable to other instruments applicable for IEF measurements.

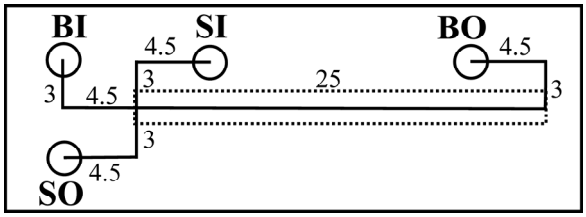


Figure 11. Design of the Shimadzu microchip. Dotted rectangle represents the part of the separation channel imaged by the detector. Inner walls of the channels are covered by linear polyacrylamide to suppress the EOF. Lengths of the channels are given in mm. Reservoirs: buffer inlet (BI), buffer outlet (BO), sample inlet (SI), sample outlet (SO).

Three therapeutic proteins (hirudin, erythropoietin (EPO), and bevacizumab, respectively) have been successfully focused on the chip using MCE-2010 apparatus (see Fig. 4 in **P4**) and the results have been compared to the results of standard cIEF (see Tab. 1 in **P4**). Well comparable peak profiles and pI values have been obtained for hirudin as well as for bevacizumab using both techniques. Erythropoietin under non-denaturing conditions (without addition of urea) was successfully focused only on the microchip. Nevertheless, its peak profile and the calculated pI values are in good agreement with published data. The reproducibility of the determination of pI values is somewhat lower on the microchip in comparison with cIEF. On the other hand, the chip

measurement is much faster, as it does not require the tedious mobilization step (unlike conventional cIEF).

4.4.2. MC-IEF applications

An overview of all available MC-IEF applications from the literature is given in **P5** in the Appendix, section 7.3. The feasibility of performing IEF on microchip has been demonstrated and MC-IEF has become a new emerging analytical technology with a number of applications. Compared to miniaturization of other electrophoretic techniques, the adaptation of IEF to microchip has several advantages. First, the resolution of IEF separation is independent of the separation length, which is a unique feature among the electrophoretic separation techniques. Second, the problematic cross injection commonly employed for electrophoresis miniaturized to the chip is avoided in case of MC-IEF. Finally, the planar arrangement of the microchip facilitates the implementation of WCID, which is the most suitable detection for IEF separation.

MC-IEF has been successfully coupled to another separation technique as well as to MS detection, which clearly demonstrates the potential of this technique for development of integrated μ-TAS. In addition to standard arrangement of MC-IEF separation, various non-standard approaches to MC-IEF have been developed. The alternative approaches to MC-IEF differ in terms of unusual sample introduction, kinds of support used for IEF separation (see Fig. 2 in **P5**), and in the manner in which the pH gradient is generated (see Fig. 3 in **P5**).

4.4.2.1. Pharmaceutical applications of MC-IEF

Protein medications are the most rapidly expanding class of therapeutics, serving nowadays patients with broad range of diseases [78]. Consequently, suitable analytical methods for characterization of active proteins and for quality control of drug substances as well as drug products are essential in the pharmaceutical industry from the development through final lot manufacturing to ensure the pharmaceutical efficacy and safety of the protein-based drugs [79]. IEF serves for separation of proteins and peptides on the basis of their isoelectric points and can therefore be applied for testing the

identity, purity, size/charge heterogeneity, as well as the stability of active proteins in pharmaceutical formulations.

The monoclonal antibodies (MAB) produced from a single cell clone are initially homogeneous but in fact they are heterogeneous due to various enzymatic and nonenzymatic modifications taking place after the synthesis. The most common modifications result from incomplete formation of disulfide bonds, glycosylation, N-terminal pyroglutamine cyclization, C-terminal lysine processing, deamidation, isomerization, and oxidation [80]. Most of these modifications can be monitored by IEF as they induce a shift in the pI value of the individual isoforms.

In cooperation with a pharmaceutical company (Solvias AG), the practical applicability of our methodology for MC-IEF performed on MCE-2010 apparatus has been tested for characterization of some protein-based drugs. For this purpose, the charge heterogeneity of two MAB has been investigated using MC-IEF and the peak profiles as well as the calculated pI values have been compared to standard cIEF method (see Tab. 3). The MC-IEF electropherograms of the investigated MAB along with the identification of the source of heterogeneity are given in Fig. 12.

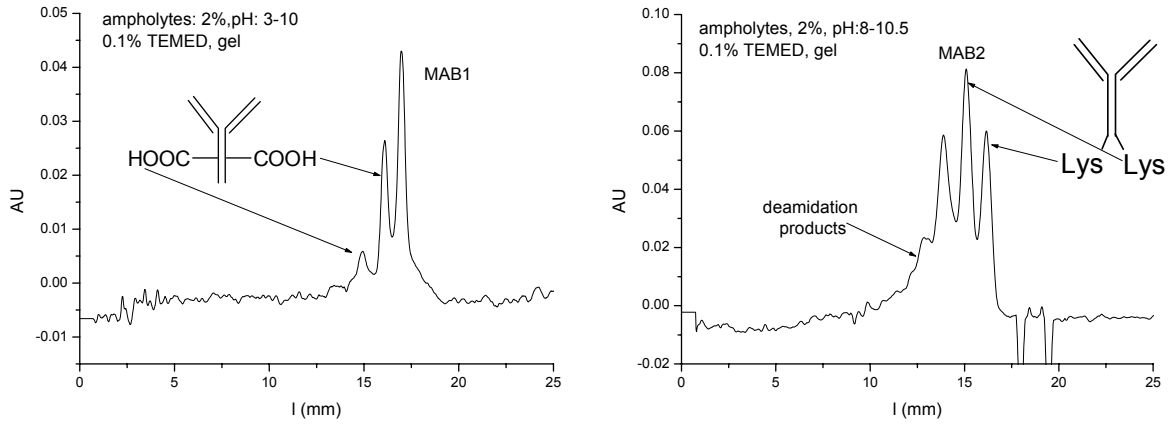


Figure 12. The MC-IEF electropherograms of two monoclonal antibodies (MAB1, MAB2) obtained using MCE-2010 instrument. Ampholytes from Fluka. Anolyte: 10 mM phosphoric acid in water, catholyte 20 mM NaOH in water. Focusing time: MAB1: 560 s; MAB2 460 s. Voltage: BI=1.8 kV, SI=SO=1.3 kV, BO=0 kV. Other conditions are given in the Figure.

Deamidation of side chains of asparagine and glutamine residues has been identified as a source of charge heterogeneity of MAB1. Since an additional charge is introduced to the protein per one deamidation, two deamidation products derived from MAB1 have been detected (Fig. 12, left). An incomplete removal of C-terminal lysine residues from immunoglobulin G heavy chains is the cause of charge heterogeneity of MAB2, where variants containing none, one, and two lysine residues have been separated by MC-IEF (Fig. 12, right). In addition to the three main peaks, small peaks resulting from deamidation of the MAB2 protein have been detected as well.

Charge heterogeneity of proteins variants can also result from modifications in the glycosylation. Variations in the oligosaccharide chain structure present on glycoproteins can significantly affect many protein properties such as solubility, specific activity, circulatory half-life, antigenicity, resistance to protease attack, and thermal denaturation [79]. On the example of a glycoprotein EPO, the effect of stressed conditions (kept at pH 3 and temperature 40 ºC for 166 hours) on the glycoforms' profile was investigated. In contrast to previous MC-IEF separation of EPO under non-denaturing conditions (see Fig. 4b in **P4**), a urea has been added for MC-IEF measurement at this time in order to enable a direct comparison of the results with standard cIEF (where the IEF separation under non-denaturing conditions failed). The pI values of non-stressed EPO from the MC-IEF separation have again been compared to standard cIEF (see Tab. 3). The comparison of electropherograms of EPO as well as stressed EPO is depicted in Fig. 13. The treatment of EPO with an acid under elevated temperature has led to hydrolyzation of sialic acid residues and, thus, to a marked shift in pI values of the isoforms. Evidently, MC-IEF can also be successfully used for monitoring of the stability of protein-based drugs.

The overview of the pI values of EPO, MAB1 and MAB2 calculated from MC-IEF measurements as well as from cIEF measurements, along with the relative standard deviations (RSD), is given in Table 3. Even though the reproducibility of pI values determination from MC-IEF separations is somewhat lower than in case of cIEF, the calculated pI values are well comparable, which supports the credibility of the MC-IEF measurements. A clear advantage of MC-IEF over cIEF is the short separation time (4-7 min compared to up to 60 min using cIEF).

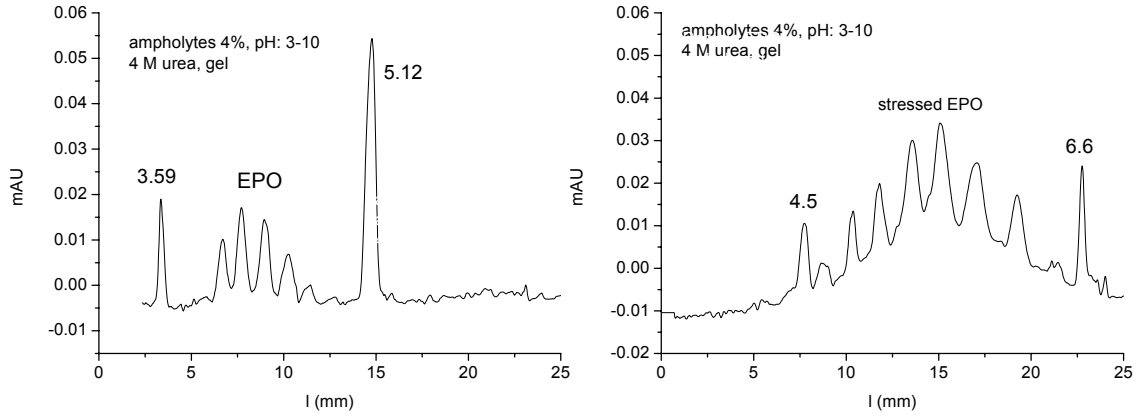


Figure 13. The MC-IEF electropherograms of EPO and stressed EPO obtained using MCE-2010 instrument. Ampholytes from Fluka. Anolyte: 10 mM phosphoric acid in water, catholyte 20 mM NaOH in water. Focusing time: 240 s. Voltage: BI=1.8 kV, SI=SO=1.3 kV, BO=0 kV. Other conditions are given in the Figure.

protein	**MC-IEF**	**cIEF with chemical mobilization**
	pI (RSD%)	pI (RSD%)
Erythropoietin (EPO)	4.03 (0.4) 4.16 4.33 4.51	4.35 (0.1) 4.51 4.61 4.74
MAB1	6.05 (0.3) 6.21 6.34	5.90 (0.1) 6.00 6.12
MAB2	8.03 (0.2) 8.11 8.17	8.20 (0.1) 8.33 8.42

Table 3. Comparison of the pI determination by MC-IEF and conventional cIEF. The RSD were calculated from 4 measurements (n = 4).

The results summarized in this subchapter demonstrate that MC-IEF performed on MCE-2010 can be utilized effectively for characterization of protein-based drugs in pharmaceutical industry as well as for assessment of the stability of a drug substance and a drug product. MC-IEF can fearlessly compete not only with conventional cIEF but also with the iCE280 Analyzer, a commercial cIEF-WCID instrument directly developed for high-throughput IEF measurements [67,68]. The separation speed of IEF performed on MCE-2010 and iCE280 Analyzer is well comparable, as both instruments are equipped with UV imaging detector. However, MCE-2010 enables recording the image at variable detection wavelength in the range of 190-370 nm (unlike iCE280 Analyzer, which allows detection only at 280 nm) and is versatile, not restricted solely to IEF measurements. On the other hand, a mono functional iCE280 Analyzer offers a higher precision of pI values determination and might be therefore preferred by laboratories specialized on IEF measurements.

5. Conclusions

MCE was shown as promising analytical technology that can in many aspects successfully compete with traditional CE. Enormous analytical potential of MCE is distinctly demonstrated by its ultra high throughput, reduced costs, and easy integration of multiple analytical steps leading eventually to the development of a true μ-TAS. The transfer of CE to the planar microfabricated devices is now well established and the consequences of the miniaturization are well documented. Numerous strategies have been developed to overcome the potential drawbacks associated with the miniaturization process. The commercialization of easily operable disposable microchips for routine analyses can be expected in the near future, which will revolutionize the analytics in many laboratories, especially in pharmaceutics, medicine, and life sciences.

On the example of presented simultaneous separation of catecholamines and their cationic metabolites, MCE was demonstrated as an attractive tool for cost- and time-saving development of a separation method for complex sample mixtures. Moreover, MCE can also be effectively applied for highly selective and sensitive on-chip amperometric detection of catecholamines and their derivatives. The detector response on the chip has been amplified by employing an enzyme-catalyzed reaction, as well as a CNT-modified working electrode. The enhanced detection sensitivity enables direct measurement of the level of these compounds in biological samples (urine, brain samples) without any pretreatment. Furthermore, specific investigations such as affinity measurements and isoelectric focusing can be easily performed on the microchip as evidenced from the reviews on available MC-ACE as well as MC-IEF applications. Additionally, the feasibility of performing MC-IEF measurements on a commercial MCE instrument made by Shimadzu (MCE-2010) has been demonstrated for the first time. Last but not least, the practical applicability of MCE-2010 for MC-IEF of pharmaceuticals has been proved by direct comparison of MC-IEF analyses of protein-based substances and drugs with conventional cIEF analyses carried out in a pharmaceutical company.

6. References

[1] G.J.M. Bruin, Electrophoresis 21 (2000) 3931.

[2] E.S. Roddy, H.W. Xu, A.G. Ewing, Electrophoresis 25 (2004) 229.

[3] C.A. Emrich, H.J. Tian, I.L. Medintz, R.A. Mathies, Anal. Chem. 74 (2002) 5076.

[4] I.L. Medintz, B.M. Paegel, R.G. Blazej, C.A. Emrich, L. Berti, J.R. Scherer, R.A. Mathies, Electrophoresis 22 (2001) 3845.

[5] A.R. Stettler, M.A. Schwarz, J. Chromatogr. A 1063 (2005) 217.

[6] C.-X. Zhang, A. Manz, Anal. Chem. 73 (2001) 2656.

[7] H.J. Crabtree, E.C.S. Cheong, D.A. Tilroe, C.J. Backhouse, Anal. Chem. 73 (2001) 4079.

[8] S.C. Jacobson, R. Hergenroder, L.B. Koutny, R.J. Warmack, J.M. Ramsey, Anal. Chem. 66 (1994) 1107.

[9] J.P. Alarie, S.C. Jacobson, C.T. Culbertson, J.M. Ramsey, Electrophoresis 21 (2000) 100.

[10] S.C. Jacobson, L.B. Koutny, R. Hergenroder, A.W. Moore, J.M. Ramsey, Anal. Chem. 66 (1994) 3472.

[11] S.C. Jacobson, R. Hergenroder, A.W. Moore, J.M. Ramsey, Anal. Chem. 66 (1994) 4127.

[12] J.P. Kutter, R.S. Ramsey, S.C. Jacobson, J.M. Ramsey, J. Microcolumn Sep. 10 (1998) 313.

[13] N.Y. Lee, M. Yamada, M. Seki, Anal. Sci. 20 (2004) 483.

[14] F. Lacharme, M.A.M. Gijs, Electrophoresis 27 (2006) 2924.

[15] Y. Luo, D.P. Wu, S.J. Zeng, H.W. Gai, Z.C. Long, Z. Shen, Z.P. Dai, J.H. Qin, B.C. Lin, Anal. Chem. 78 (2006) 6074.

[16] L. Zhang, X.F. Yin, Z.L. Fang, Lab Chip 6 (2006) 258.

[17] H.W. Gai, L.F. Yu, Z.P. Dai, Y.F. Ma, B.C. Lin, Electrophoresis 25 (2004) 1888.

[18] G.S. Zhuang, G. Li, Q.H. Jin, J.L. Zhao, M.S. Yang, Electrophoresis 27 (2006) 5009.

[19] K.W. Ro, K. Lim, B.C. Shim, J.H. Hahn, Anal. Chem. 77 (2005) 5160.

[20] H. Salimi-Moosavi, Y.T. Jiang, L. Lester, G. McKinnon, D.J. Harrison, Electrophoresis 21 (2000) 1291.

[21] S. Gotz, U. Karst, Anal. Bioanal. Chem. 387 (2007) 183.

[22] W.R. Vandaveer, S.A. Pasas-Farmer, D.J. Fischer, C.N. Frankenfeld, S.M. Lunte, Electrophoresis 25 (2004) 3528.

[23] J. Wang, Electroanalysis 17 (2005) 1133.

[24] M.A. Schwarz, P.C. Hauser, Lab Chip 1 (2001) 1.

[25] Y. Wu, Lin, J.M., Su, R., Qu, F., Cai, Z.,, Talanta 64 (2004) 338.

[26] J.J. Xu, A.J. Wang, H.Y. Chen, TrAC, Trends Anal. Chem. 26 (2007) 125.

[27] M.A. Schwarz, B. Galliker, K. Fluri, T. Kappes, P.C. Hauser, Analyst 126 (2001) 147.

[28] T. Kappes, P.C. Hauser, Analyst 124 (1999) 1035.

[29] I.M. Lazar, J. Grym, F. Foret, Mass Spectrom. Rev. 25 (2006) 573.

[30] S.L.S. Freire, A.R. Wheeler, Lab Chip 6 (2006) 1415.

[31] Q.L. Mao, J. Pawliszyn, Analyst 124 (1999) 637.

[32] A.E. Herr, J.I. Molho, K.A. Drouvalakis, J.C. Mikkelsen, P.J. Utz, J.G. Santiago, T.W. Kenny, Anal. Chem. 75 (2003) 1180.

[33] X.Z. Wu, T.M. Huang, Z. Liu, J. Pawliszyn, TrAC, Trends Anal. Chem. 24 (2005) 369.

[34] J. Khandurina, A. Guttman, Curr. Opin. Chem. Biol. 7 (2003) 595.

[35] S.F.Y. Li, L.J. Kricka, Clin. Chem. 52 (2006) 37.

[36] H. Wisser, in L. Thomas (Editor), Labor und Diagnose: Indikation und Bewertung von Laborbefunden für medizinische Diagnostik, TH-Books, Frankfurt, Germany, 2000, p. 1062.

[37] J. Bergquist, A. Sciubisz, A. Kaczor, J. Silberring, J. Neurosci. Methods 113 (2002) 1.

[38] J.C. Alvarez, D. Bothua, I. Collignon, C. Advenier, O. Spreux-Varoquaux, Biomed. Chromatogr. 13 (1999) 293.

[39] S. Sarre, Y. Michotte, P. Herregodts, D. Deleu, N.D. Klippel, G. Ebinger, J. Chromatogr. B 575 (1992) 207.

[40] K.A. Sagar, M.R. Smyth, Journal of Pharmaceutical and Biomedical Analysis 22 (2000) 613.

[41] X.S. Zhu, P. N.; Barrett, D. A., Anal. Chim. Acta 478 (2003) 259.

[42] M.A. Fotopoulou, Ioannou, P. C., Anal. Chim. Acta 462 (2002) 179.

[43] M. Tsunoda, Anal. Bioanal. Chem. 386 (2006) 506.

[44] Z.D. Peterson, D.C. Collins, C.R. Bowerbank, M.L. Lee, S.W. Graves, J. Chromatogr. B 776 (2002) 221.

[45] L. Stoica, A. Lindgren-Sjolander, T. Ruzgas, L. Gorton, Anal. Chem. 76 (2004) 4690.

[46] F. Lisdat, U. Wollenberger, A. Makower, H. Hortnagl, D. Pfeiffer, F.W. Scheller, Biosens. Bioelectron. 12 (1997) 1199.

[47] F. Mizutani, S. Yabuki, M. Asai, Biosens. Bioelectron. 6 (1991) 305.

[48] O.D. Leite, O. Fatibello, A.D. Barbosa, J. Braz. Chem. Soc. 14 (2003) 297.

[49] T. Yao, C.O. Ho, Bunseki Kagaku 51 (2002) 469.

[50] A.L. Ghindilis, A. Makower, C.G. Bauer, F.F. Bier, F.W. Scheller, Anal. Chim. Acta 304 (1995) 25.

[51] M.A. Schwarz, Electrophoresis 25 (2004) 1916.

[52] J. Wang, Electroanalysis 17 (2005) 7.

[53] K. Wu, S. Hu, Microchim. Acta 144 (2004) 131.

[54] W. Zhang, Wan, F., Xie, Y., Gu, J.,Wang, J., Yamamoto, K, Jin, L., Anal. Chim. Acta 512 (2004) 207.

[55] W. Zhang, Y. Xie, S. Ai, F. Wan, J. Wang, L. Jin, J. Jin, J. Chromatogr. B 791 (2003) 217.

[56] M. Chicharro, A. Sanchez, E. Bermejo, A. Zapardiel, M.D. Rubianes, G.A. Rivas, Anal. Chim. Acta 543 (2005) 84.

[57] J. Wang, G. Chen, M.P. Chatrathi, M. Musameh, Anal. Chem. 76 (2004) 298.

[58] M. Pumera, X. Llopis, A. Merkoci, S. Alegret, Microchim. Acta 152 (2006) 261.

[59] C. Hu, W. Wang, K. Liao, G. Liu, Y. Wang, J. Phys. Chem. Solids 65 (2004) 1731.

[60] M. Pumera, S. Sanchez, I. Ichinose, J. Tang, Sens. Actuators, B 123 (2007) 1195.

[61] A. Guiseppi-Elie, L. Chenghong, R.H. Baughman, Nanotechnology 13 (2002) 559.

[62] L. Wang, Z. Yuan, Sensors 3 (2003) 544.

[63] D. Pan, J. Chen, S. Yao, W. Tao, L. Nie, Anal. Sci. 21 (2005) 367.

[64] F. Patolsky, Y. Weizmann, I. Willner, Angew. Chem. Int. Ed. 43 (2004) 2113.

[65] H. Xue, W. Sun, B. He, Z. Shen, Synthetic Metlas 135-136 (2003) 831.

[66] J.Q. Wu, S.C. Li, A. Watson, J. Chromatogr. A 817 (1998) 163.

[67] X.Z. Wu, J.Q. Wu, J. Pawliszyn, LC-GC 19 (2001) 527.

[68] N. Li, K. Kessler, L. Bass, D. Zeng, J. Pharm. Biomed. Anal. 43 (2007) 963.

[69] W. Tan, Z.H. Fan, C.X. Qiu, A.J. Ricco, I. Gibbons, Electrophoresis 23 (2002) 3638.

[70] J. Han, A.K. Singh, J. Chromatogr. A 1049 (2004) 205.

[71] C. Das, Z.H. Fan, Electrophoresis 27 (2006) 3619.

[72] O. Vesterbe, H. Svensson, Acta Chem. Scand. 20 (1966) 820.

[73] F. Kitagawa, S. Aizawa, K. Otsuka, Anal. Sci. 21 (2005) 61.

[74] M. Ludwig, F. Kohler, D. Belder, Electrophoresis 24 (2003) 3233.

[75] E. Guihen, G.D. Sisk, N.M. Scully, J.D. Glennon, Electrophoresis 27 (2006) 2338.

[76] T. Miyado, Y. Tanaka, H. Nagai, S. Takeda, K. Saito, K. Fukushi, Y. Yoshida, S. Wakida, E. Niki, J. Chromatogr. A 1109 (2006) 174.

[77] Z.Q. Xu, Y. Nakamura, T. Hirokawa, Electrophoresis 26 (2005) 383.

[78] B. Leader, Q.J. Baca, D.E. Golan, 7 (2008) 21.

[79] K. Ahrer, A. Jungbauer, J. Chromatogr. B 841 (2006) 110.

[80] H. Liu, G. Gaza-Bulseco, D. Faldu, C. Chumsae, J. Sun, J. Pharm. Sci. (2008) doi:10.1002/jps.21180.

7. Appendix

7.1. List of contributions

7.1.1. Publications (those marked **bold** are cited in this thesis)

1. Barták, P., Šimánek V., Vlčková, M., Ulrichová, J.,Vespalec, R.: Interactions of sanguinarine and chelerythrine with molecules containing mercapto group, J. Phys. Org. Chem. 2003, 16 (10), 803-810.
2. Vespalec, R., Barták, P., Šimánek V., Vlčková, M.: Electrophoretic Investigation of interactions of sanguinarine and chelerythrine with molecules containing mercapto group, J. Chromatogr. B 2003, 797 (1-2), 357-366.
3. Vlčková, M., Barták, P., Kubáň, V.: Electrophoretic studies of acid-base properties of sanguinarine and chelerythrine alkaloids, J. Chromatogr. A 2004, 1040 (1), 141-145.
4. Vespalec, R., Vlčková, M., Horáková, H.: Aggregation and other intermolecular interactions of biological buffers observed by capillary electrophoresis and UV-photometry, J. Chromatogr. A 2004, 1051 (1-2), 75-84.
5. Vespalec, R., Vlčková, M., Kubáň, V.: Effects of the limited analyte solubility on its mobility and zone shape: Electrophoretic behavior of sanguinarine and chelerythrine around pH 7, Electrophoresis 2005, 26 (17): 3265-3272.
6. Vlčková, M., Kubáň, V., Vičar, J., Šimánek, V.: Capillary zone electrophoretic studies of interactions of some quaternary isoquinoline alkaloids with DNA constituents and DNA, Electrophoresis 2005, 26 (9): 1673-1679.
7. **Vlčková, M., Schwarz, M.A.: Enzymatic sensitivity enhancement of biogenic monoamines on a chip, Electrophoresis 2005, 26 (14), 2701-2707.**
8. **Vlčková, M., Stettler, A., Schwarz M.A.: Microchip Affinity Capillary Electrophoresis Applications and Recent Advances, J. Liq. Chromatogr. Relat. Technol. 2006, 29 (7-8), 1047-1076.**

9. **Vlčková, M., Schwarz, M.A.: Determination of cationic neurotransmitters and metabolites in brain homogenates by microchip electrophoresis and carbon nanotube-modified amperometry, J. Chromatogr. A 2007, 1142 (2), 214-221.**
10. **Vlčková, M., Kalman, F., Schwarz, M.A.: Pharmaceutical applications of isoelectric focusing on microchip with imaged UV detection, J. Chromatogr. A 2008, 1181, 145-152.**
11. **Vlčková, M., Schwarz, M.A.: Microchip isoelectric focusing applications, accepted, Chimia 2008.**

7.1.2. Oral presentations and posters (presenting author marked **bold**)

Oral presentations:

Vlčková, M., Vespalec, R.: Studies of electrophoretic properties of sanguinarine and chelerythrine (in Czech); Proceedings from 30th Conference on Synthesis and analysis of drugs, 2001, Brno, Czech Republic.

Vlčková, M., Barták, P., Vespalec, R.: Electrophoretic behaviour and properties of sanguinarine and chelerythrine; 14th International Conference Chromatographic methods and Human Health Proceedings, 12.-15.11.2001, Piešťany, Slovak Republic.

Vlčková, M., Barták, P., Vespalec, R.: pK determination of sparingly soluble weak bases; Proceedings from the Conference on Advances in chromatography and electrophoresis & Chiranal 2002, 24.-27.6.2002, Olomouc, Czech Republic.

Vlčková, M., Schwarz, M.A.: Sensitive enzyme based amperometric detection of neurotransmitters on a chip, The abstract book of the 4th International Symposium on Separations in BioSciences, 18.-21.9.2005, Utrecht, The Netherlands.

Vlčková, M., Schwarz, M.A.: Sensitive determination of neurotransmitters and their metabolites in biological samples using microchip electrophoresis, The abstract book of the 5th Swiss Snow symposium, 2.-4.3.2007, Fiesch, Switzerland.

Vlčková, M., Schwarz, M.A.: Microchip electrophoresis bioanalytical applications, The abstract book of the international symposium Advances in Chromatography and Electrophoresis 2007 & Chiranal 2007, 24.-27.6.2007, Olomouc, Czech Republic.

Vlčková, M., Schwarz, M.A.: Microchip electrophoresis bioanalytical applications, Abstracts from Fall meeting of the Swiss chemical society 2007, Chimia 2007, 61 (7/8), 418, 12.9.2007, Lausanne, Switzerland.

Posters:

Vlčková, M., Barták, P., Vespalec, R.: Determination of pK values of alkaloids sanguinarine and chelerythrine by capillary zone electrophoresis, Abstracts from the 54th Congress of Chemical Societies, Chemické Listy 2002, 96, S196-S197; 30.6.-4.7.2002, Brno, Czech Republic.

Vlčková, M., Barták, P., Vespalec, R.: Knowledge from measurement of pK values of alkaloids sanguinarine and chelerythrine by capillary zone electrophoresis, Advance in separation sciences on the day before the 100 years anniversary of chromatography disover, The abstract book of the 8th International Symposium on Separation Sciences, 8.-12.9.2002, Toruń, Poland.

Vlčková, M., Barták, P., Vespalec, R.: Determination of pK_{R+} constants and water-solubility of alkaloids sanguinarine and chelerythrine by capillary zone electrophoresis, Sigma-Aldrich Conference Abstracts, Chemické Listy 2003, 97, 319; 4.-7.6.2003, Devět Skal, Žďárské vrchy, Czech Republic.

Bolyan, D., Kalman, F., Schwarz, M.A., **Vlčková, M.:** Comparison of conventional capillary IEF and microchip IEF equipped with an imaged detection system for the analysis of therapeutic proteins, The abstract book of the CE in the Biotechnology and Pharmaceutical Industries: 9th symposium on the Practical applications for the analysis of proteins, nucleotides and small molecules, 14.-18.10.2007, Miami, Florida, U.S.A.

7.2. Curriculum vitae

Name:	Markéta Vlčková
Born:	28th May 1977
Citizenship:	Czech Republic
Marital status:	single

Qualifications:

2004 – till now	Assistant/PhD. student in the Department of Chemistry, University of Basel, Switzerland
1995 – 2000	MSc. degree, Faculty of Chemistry, Department of Food Chemistry and Biotechnology, Brno University of Technology, Czech Republic

Summary of praxis:

2002 – 2004	Research worker in the Department of Chemistry and Biochemistry, Mendel University of Agriculture and Forestry, Brno, Czech Republic
2000 – 2002	Research worker in the Institute of Analytical Chemistry, Academy of Sciences of the Czech Republic, Brno, Czech Republic

Research and work activities:

Microchip electrophoresis bioanalytical applications – dissertation thesis

Teaching students in theoretical and practical courses in analytical chemistry (theoretical calculations, CE, IEC)

Investigation of interactions of alkaloids with DNA and DNA constituents by CE

Development of a method for isolation and characterization of peptides from various types of cheeses – diploma thesis (used techniques: HPLC, SEC and UV/VIS spectrophotometry)

Presentations:

4 posters and 7 oral presentations on the international scientific meetings, co-author of 11 publications

Awards:

2000	Master's diploma with distinction
2002	Presentation award of the Institute of analytical chemistry for attending a conference (8th International Symposium on Separation Sciences, Toruń, Poland)
2003	Award for attending 5th Interdisciplinary Conference for Young Biologists, Biochemists and Chemists paid by Sigma-Aldrich
2007	Best oral presentation (Mettler Toledo award)

Certificates:

Zertifikat Deutsch als fremd Sprache (1995)

Certificate in chemometrics (2004)

Other skills:

Fluent knowledge of German, C1/C2 level

Fluent knowledge of English, C1/C2 level

Conversational knowledge of French, B1/B2 level

Driving licence B

User of PC (Windows, Office, Internet, Isis Draw, Photoshop)

Hobbies:

Reading books, travelling, playing piano, electronic music, sports (aerobics, fitness).

7.3. Enclosed publications

P1 Vlčková, M., Schwarz, M.A.: Determination of cationic neurotransmitters and metabolites in brain homogenates by microchip electrophoresis and carbon nanotube-modified amperometry, J. Chromatogr. A 2007, 1142 (2), 214-221.

P2 Vlčková, M., Schwarz, M.A.: Enzymatic sensitivity enhancement of biogenic monoamines on a chip, Electrophoresis 2005, 26 (14), 2701-2707.

P3 Vlčková, M., Stettler, A., Schwarz M.A.: Microchip Affinity Capillary Electrophoresis Applications and Recent Advances, J. Liq. Chromatogr. Relat. Technol. 2006, 29 (7-8), 1047-1076.

P4 Vlčková, M., Kalman, F., Schwarz, M.A.: Pharmaceutical applications of isoelectric focusing on microchip with imaged UV detection, J. Chromatogr. A 2008, 1181, 145-152.

P5 Vlčková, M., Schwarz, M.A.: Microchip isoelectric focusing applications, Chimia 2008, 62, 244-248.

Publication 1

Determination of cationic neurotransmitters and metabolites in brain homogenates by microchip electrophoresis and carbon nanotube-modified amperometry

Vlčková, M., Schwarz, M.A.

J. Chromatogr. A 2007, 1142 (2), 214-221

Available online at www.sciencedirect.com
ScienceDirect
Journal of Chromatography A, 1142 (2007) 214–221
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Determination of cationic neurotransmitters and metabolites in brain homogenates by microchip electrophoresis and carbon nanotube-modified amperometry

Markéta Vlčková, Maria A. Schwarz *

Department of Chemistry, University of Basel, Spitalstrasse 51, 4056 Basel, Switzerland

Received 2 November 2006; received in revised form 1 December 2006; accepted 11 December 2006
Available online 17 December 2006

Abstract

An electrophoretic method for simultaneous determination of catecholamines and their *O*-methoxylated metabolites on the microchip as well as in the capillary is presented. A complex separation system employing sodium dodecyl sulfate (SDS) micelles, dendrimers forming a second pseudostationary phase and borate complexation is needed for the satisfactory separation of the selected compounds on the short migration length. A carbon nanotube-modified working electrode has been applied for the sensitive amperometric detection with submicromolar detection limits. The applicability of this new method for the analytics of real samples is demonstrated by analysis of mouse brain homogenate on the microchip and human urine by capillary electrophoresis.

Keywords: Neurotransmitter; Metabolite; Microchip; Brain homogenate; Carbon nanotube; Amperometry

1. Introduction

The measurement of the level of catecholamines (dopamine, noradrenaline, and adrenaline) in biological fluids has an essential role in the diagnostics of diseases. In particular, various tumours of the sympatho-adrenal system, such as pheochromatocytoma or neuroblastoma are diagnosed by determination of the catecholamines and/or their metabolites in urine and blood [1]. Furthermore, the measurement of the level of catecholamines and their metabolites in the cerebrospinal fluid (CSF) is used in diagnostics of hypertension, multiple sclerosis, or Parkinson's disease [2]. In animal models, the measurement of these compounds in the brain homogenates is an important research strategy for studying the role of the autonomic nervous system in various physiological or pathophysiological conditions [3]. Last but not least, the quantification of catecholamines and their metabolites in various biological samples is also very useful for the investigation of drug action on the dopaminergic system.

The prevailing analytical method employed for quantitative determination of these compounds is high-performance liquid chromatography (HPLC) equipped with sensitive electrochemical [4,5] or fluorimetric detection [6,7]. Although HPLC provides low detection limits and good reproducibility, it suffers from cost, labour, and long analysis times. Moreover, the purification and preconcentration of the sample is often required prior to analysis [2,8].

Capillary electrophoresis (CE) often allows faster analyses than HPLC, but its applicability for the analysis of complex mixtures of catecholamines and/or their metabolites in biological fluids is limited by lower detection limits and similar electrophoretic behaviour of these compounds. Despite numerous reports on this topic available in the literature, see e.g. Refs. [9–12], many applications are restricted to a few compounds of the complex mixture present in biological samples. Structurally similar compounds, such as catecholamines [9,13], cationic metabolites [11], or anionic metabolites (e.g. vanillylmandelic acid or homovanillic acid) [14,15], are often analysed independently or particular analytes of interest are selected from the complex biological samples for their determination [10,12,16]. Regardless of the selection of the compounds for analysis, a careful adjustment of the separation conditions is required to

* Corresponding author. Tel.: +41 61 2671002; fax: +41 61 2671005.
E-mail addresses: Maria.Schwarz@unibas.ch, Marketa.Vlckova@unibas.ch (M.A. Schwarz).

0021-9673/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2006.12.040

achieve a successful separation, see e.g. Refs. [9,10]. The addition of various modifiers is frequently used to alter the separation selectivity. For example, borate complexation can be applied to improve the separation of these substances [10,12,13,15,17]. The selectivity could be strongly altered, and thus the separation substantially improved, by using a micellar system (micellar electrokinetic capillary chromatography, MEKC) mostly by employing sodium dodecyl sulfate (SDS) micelles [12,15,16,18]. In some reports, the effects of micelles and borate were combined to improve the separation and resolution [12,15].

The detection of the biogenic amines is also challenging in the CE analytics of catecholamines and/or their metabolites in biological samples, because their levels in biological fluids are very low (up to the subnanomolar range). Although UV detection has been successfully utilized for the detection of the catecholamines in urine after a proper pretreatment of the sample [10,15,19], other detection methods with better sensitivity have been investigated. Sensitive detection of catecholamines and related compounds has been achieved by employing fluorescence [20,21], luminescence [17], mass spectrometry [11], or most commonly by electrochemical detection [9,12,16,18]. Among these sensitive detection methods, electrochemical detection is the simplest one since it does not require the derivatization of the analytes (unlike fluorescence or luminescence) or expensive equipment (unlike mass spectrometry). However, electrochemical detection is not sensitive enough for the detection of catecholamines and their metabolites in common biological samples without sample preconcentration or other strategy to improve the detection sensitivity [9]. The limits of detection (LODs) for these compounds have been improved, e.g. by employing parallel-opposed dual-electrode amperometric detector, where analyte cycles between two working electrodes [22], or by field-amplified sample stacking [9].

Another attractive strategy for lowering LOD is to apply enzymes, which enable cycling of the analyte between enzyme and electrode. Glucose oxidase has been successfully applied for sensitive detection of catecholamines after their electrophoretic separation on microchip [23]. The combination of the glucose oxidase with NADH made it possible to detect the catecholamines even in low nanomolar range [24]. A novel approach to lower the LOD of these compounds represents the use of carbon nanotube (CNT)-modified electrodes for amperometric detection in CE [25] or microchip electrophoresis [26,27]. However, except for dual-electrode amperometric detection [22] and field amplified sample stacking [9] applied for urine analysis of catecholamines and related compounds, none of these detection improvement strategies in CE has been tested for the analysis of real samples.

It should also be noted that electrophoresis applied up to now for the analytics of biogenic monoamines in biological samples has been almost exclusively performed in the capillary format. To our knowledge, only two reports of the application of the microchip or a capillary with miniaturized separation length for determination of dopamine in CSF (using electrochemical detection) [28] and in the brain dialysate (using fluorescence detection) [29] are available in the literature. In both studies, only dopamine has been measured, either as a reference molecule or as an analyte. Using fluorescence detection and by application of SDS-micelles and cyclodextrins as a separation medium, more than 60 peaks of unknown identity have been electrophoretically separated and detected [29].

The aim of the present study is to develop a sensitive and selective method for the simultaneous determination of catecholamines and their cationic metabolites in biological samples by electrophoresis on a microchip. All these compounds occur in the biological samples and therefore their simultaneous determination without mutual interference is of importance for diagnosis of the diseases and for monitoring of the drug's action. Additionally, our new separation and detection method is transferred to the application in the capillary format. The applicability of the new method is demonstrated by the analysis of the abovementioned compounds in mouse brain homogenate and in human urine.

2. Experimental

2.1. Chemicals

The standards of dopamine (D), adrenaline (A), noradrenaline (NA), and methoxytyramine (MT) were purchased from Fluka (Buchs, Switzerland), normetanephrine (NME) and metanephrine (ME) from Sigma–Aldrich (St. Louis, MO, USA). Sodium dodecyl sulfate and polyamidoamine (PAMAM) dendrimer (with ethylendiamine core), generation 1.5 (G1.5) were from Sigma–Aldrich and *N*,*N*′-dimethylformamide (DMF) was from Acros (NJ, USA). Multi-wall carbon nanotubes (MWCNTs) with the outer diameter 10–30 nm and length 1–2 μm were obtained from Nanostructured & Amorphous Materials (Los Amos, NM, USA). All other chemicals were of analytical grade purity. Deionized water was used throughout the study.

2.2. Apparatus

2.2.1. Microchip

The microchip employed for the electrophoretic separations in microscale format was made from glass and was purchased from Micralyne (model MC-BF4-TT100, Edmonton, Canada). The separation channel length of the microchip is 90.28 mm (80.89 mm from intersection) with a semicircular cross-section of 50 μm width and 20 μm depth. The intersection is double T-shaped with 100 μm distance between the inlet and outlet part of the injection channel. Pipette tips, serving as reservoirs for buffer and sample, were glued to the top of the chip with an epoxy adhesive (Epo-Tek OG 116, Polyscience, Baar, Switzerland). The chip was mounted on an inverse microscope (model DM IL, Leica, Basel, Switzerland) equipped with a XYZ-micromanipulator holding the working electrode connected to a home-built amperometric detector circuitry [30], which allows us to set the potential of the working electrode. A two-electrode configuration consisting of an electrophoretic counter electrode and a working electrode was employed. The platinum electrophoretic ground electrode provides a sufficiently stable potential and served therefore as a pseudoreference electrode [31]. The working electrode was attached to the micro-

manipulator and during detection its tip was positioned at the endpoint of the separation channel. Injection and separation voltage were produced by two high-voltage power supplies (model CZE1000R, Spellman, Pulborough, UK).

2.2.2. CZE

A Crystal CE Model 310 (ATI Unicam, Cambridge, UK) capillary electrophoresis apparatus with the Winprince 6.0 software control (PrinCE Technologies, Emmen, The Netherlands) was employed for the measurements in the capillary made of fused silica (50 μm i.d., 360 μm o.d., length 70 cm, BGB Analytik, Böckten, Switzerland). The simplified two-electrode amperometric detection was realized via wall-jet arrangement without decoupler using a special cell holding the capillary and both electrodes [32]. The distance between the end of the capillary and the working electrode was set by using a plastic sheet of the thickness of 30 μm as temporary spacer while assembling the cell. A platinum electrode, which represents pseudoreference, counter, and electrophoretic ground electrode in one, was positioned perpendicularly to the capillary and working electrode.

The possible interferences of amperometric detection have been avoided by placing of the detector inside a Faraday cage.

2.3. Working electrode preparation

A Teflon-coated gold wire (bare wire diameter 75 μm, coated wire diameter 112 μm, Advent Research Materials, UK) bent into the L-shape and soldered to the insulated cable served as a working electrode on the microchip and as a base for preparing of CNT-modified electrode for microchip. For the amperometric detection on the capillary, a gold wire (diameter 90 μm, Fine Wire Co., Grover Beach, CA, USA) was threaded into an electrode body consisted of a piece of fused silica capillary of 100 μm i.d. and 360 μm o.d. An insulated cable was soldered to the back end for electric contact. Epoxy glue was applied to fix the wire at both ends of the electrode body and to seal the soldier point. At the electrode tip, the wire was cut off and polished with a polishing sheet and the entire electrode was used as a base for preparing CNT-modified electrode.

MWCNTs for covering of both gold electrodes were functionalised in the first step by refluxing for 5 h in concentrated nitric acid [33,34]. The functionalisation leads to the formation of carboxyl moieties on the surface of the nanotubes [35]. Simultaneously, this treatment leads to the purification of CNTs by removal of metallic impurities [26]. Two milligrams of functionalised CNTs was then dissolved in 1 ml of *N*,*N*′-dimethylformamide (DMF) [27,36] with the help of ultrasonication. The modification of the electrode by CNTs was then accomplished by dipping of the freshly cut or polished Au electrode into the CNT suspension followed by drying at 100 °C. The dipping and drying was repeated 10 times in each case.

2.4. Electrophoretic procedure and samples

The channels of the glass microchip as well as the capillary were treated before use by rinsing with 0.1 M NaOH (10 min) followed by deionised water (10 min). The background electrolyte for separation of catecholamines and their metabolites on the microchip consisted of 5 mM borate adjusted by phosphoric acid to pH 7 with the addition of 10 mM SDS and 5 μl/ml PAMAM dendrimer G1.5. For the separation in the capillary the concentration of SDS was increased to 20 mM. The background electrolyte was used for preparing of standard sample mixtures. On the microchip, the injection was electrokinetic with injection voltage of 1 kV for 3 s; the separation voltage was then 3 kV. In the capillary, the injection was hydrodynamic with the pressure of 250 mbar for 6 s; the separation voltage there was 20 kV.

Brain homogenates were prepared by standard procedures described elsewhere, see e.g. Ref. [4]. Briefly, brain tissues were weighed frozen and were homogenized in 0.2 M $HClO_4$ containing 0.1% of Na_2EDTA. The homogenate was centrifuged to remove the precipitated protein and cell debris. The supernatant was then diluted with the running buffer and directly injected.

The spot urine was collected from a healthy volunteer and immediately analysed by CE after its dilution with 0.2 M $HClO_4$ containing 0.1 % of Na_2EDTA in order to prevent the oxidation of the analytes followed by filtration of the sample.

3. Results and discussion

3.1. Development of the method for the simultaneous separation of catecholamines and their cationic metabolites

The separation of catecholamines (dopamine, noradrenaline, and adrenaline and their *O*-methoxylated metabolites, namely 3-methoxytyramine, normetanephrine, and metanephrine is a demanding task due to their similar structure (Fig. 1(a)) and therewith nearly identical electrophoretic behaviour. Without additives, these compounds are not electrophoretically separable (Fig. 1(b)), especially if short migration lengths are used. For example, on a migration length of about 8 cm and with a moderate electroosmotic flow (EOF) a time window of less than 40 s is available for detection of cations. Moreover, if only catecholamines or only cationic metabolites are considered as analytes in the development of the separation method, different migration of these compounds cannot be guaranteed (following from the similar structure and similar electrophoretic behaviour, as demonstrated in Fig. 1(a and b). Only experiments that involve all six compounds can exclude the possible co-migration and thus interference in the real samples.

To our knowledge, there is merely one publication describing the simultaneous electrophoretic separation of all six cationic compounds (three catecholamines and their *O*-methoxylated metabolites) reported by Peterson et al. [37]. The separation was realized on long coated capillaries (polyvinyl alcohol) coupled with time of flight-mass spectrometric detection (TOF-MS). Despite low pH and coating of the capillary surface resulting in diminished EOF, the resolution between cationic amines is not sufficient for a complete separation within short separation lengths. Furthermore, the laborious coating procedure is hardly realizable with the microchips. Therefore, we followed another separation strategy based on the partition between the micellar and aqueous phase using sodium dodecyl sulfate, which has already been applied to the separation of some of the selected compounds, see e.g. Refs. [12,16].

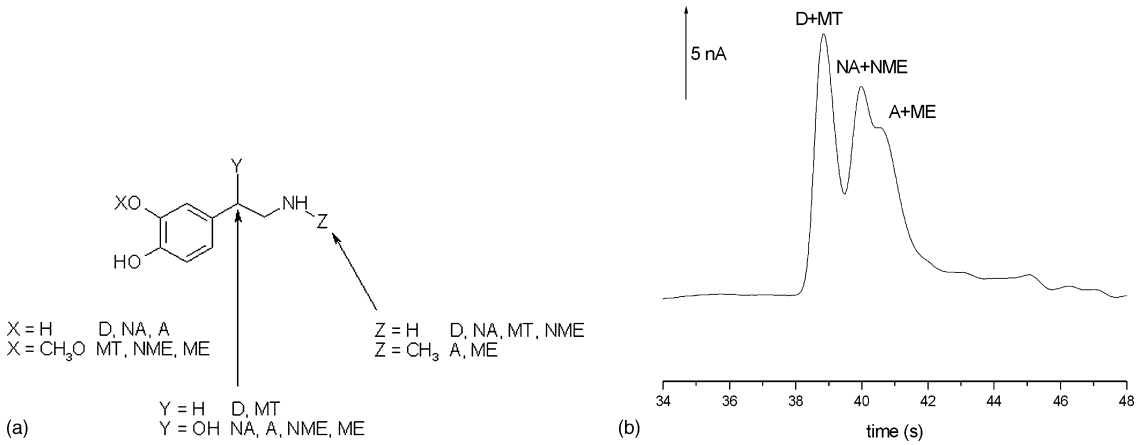


Fig. 1. (a) Chemical structure of catecholamines (D, NA, A) and their *O*-methoxylated metabolites (MT, NME, ME). (b) The electrophoretic separation of selected compounds on the microchip without any additives. Buffer: 10 mM TES–Na buffer (pH 7), separation voltage: 3 kV, injection voltage: 1 kV (3 s), Au electrode, and detection potential: 1200 mV.

The development of the separation method is summarized in Fig. 2 and is described in the following text. The catecholamines separated well on the microchip using sodium TES (*N-tris*(hydroxymethyl)methyl-2-aminoethanesulfonic acid) buffer of pH 7 [16] with the addition of 10 mM of SDS. However, NME and ME co-eluted under these conditions with A and D, respectively (Fig. 2, line 1). Unfortunately, higher concentrations of SDS could not be employed on the microchip because the overall charge of the analytes becomes too negative for their successful electrokinetic injection. Furthermore, the increased electrophoretical current is unfavourable for the detection sensitivity. Therefore, we tried to improve the separation by adding borate [12], because it forms complexes with catecholamines, but not with the *O*-methoxylated metabolites.

Surprisingly, the presence of borate (5 mM) has no, or only a slight, influence on migration of catecholamines in the TES buffer with or without the presence of SDS. Apparently, TES suppresses the complex formation; most probably this arises from some side interaction with the analytes. On the other hand, the measurements with sodium phosphate buffer at the same pH and same concentration of borate (5 mM) caused slower migration of all catecholamines indicating their complexation with borate, even though their peaks were not sufficiently resolved (Fig. 2, line 2).

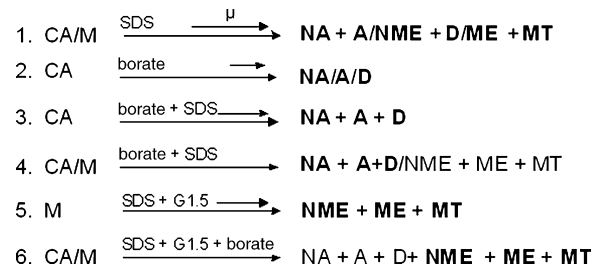


Fig. 2. Scheme of the observed shifts in migration times induced by various modifiers dissolved in the background electrolyte, CA (catecholamines): D, NA, A; M (metabolites): MT, NME, ME; left side: injected compounds, right side: detected compounds, bold indicates the analyte molecules affected by the modifier.

The addition of borate in the presence of SDS micelles had the opposite effect on the migration of the catecholamines. The borate–catecholamine complexes are more hydrophilic than NT themselves and are therefore retained to a smaller extent in the micelles (Fig. 2, line 3). However, the separation of the three catecholamines and three metabolites could not be achieved by simple adjusting of the concentration of both additives because the peaks of D and NME in this separation system overlapped (Fig. 2, line 4). In an attempt to resolve all the selected compounds, we tried to add a further modifier in addition to borate and SDS.

Various complexation agents being reported for the interaction with these compounds [38,39] were tested for this purpose. Whereas addition of cyclodextrins and crown ethers was not helpful, addition of PAMAM dendrimer of generation 1.5 made it possible to separate the metabolites from the catecholamines. The dendrimers form a second pseudostationary phase and in the combination with SDS micellar phase retain especially the metabolites, which results in their later detection times (Fig. 2, line 5). The 5 mM borate–phosphate buffer containing 10 mM SDS, and 0.5% (v/v) dendrimer PAMAM G1.5 has finally led to a satisfactory separation of all catecholamines and their *O*-methoxylated metabolites on the microchip (Fig. 2, line 6; Fig. 3). Even though the peak shape and the separation efficiency are not ideal, all peaks are sufficiently separated and easily detectable. The unfavourable peak shapes may be ascribed to the processes taking place during the separation (including the partition between aqueous and micellar phase, and further complex equilibria), which induce mismatching conductivity between sample and buffer zone and lead thus to the distortion of the peak shapes.

This separation system (5 mM borate–phosphate buffer, 10 mM SDS and 0.5% (v/v) dendrimer PAMAM G1.5) was then successfully applied to the separation of cationic amines by capillary electrophoresis. As the hydrodynamic injection is

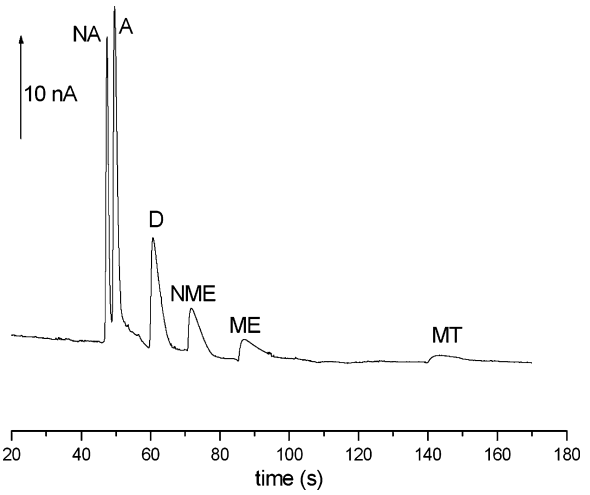


Fig. 3. The electrophoretic separation of catecholamines and cationic metabolites on the microchip. Buffer: 5 mM borate–phosphate buffer (pH 7) containing 10 mM of SDS and 5 μl/ml of PAMAM dendrimer G1.5, separation voltage: 3 kV, injection voltage: 1 kV (3 s), Au electrode, and detection potential: 1400 mV.

applicable for CE, we improved the resolution between NA and A by increasing the SDS concentration to 20 mM. The optimised separation methods for microchip as well as CE were further subjected to the development of sensitive amperometric detection of the catecholamines and their *O*-methoxylated metabolites.

3.2. Development of the method for sensitive amperometric detection of catecholamines and their cationic metabolites

Very sensitive amperometric detection of catecholamines and related compounds on the microchip has been reported by using of glucose oxidase enzyme as a buffer additive [23]. However, our measurements revealed that the complex separation system needed for the separation of selected compounds has a detrimental effect on the glucose oxidase activity. The glucose oxidase undergoes a quick oxidation in the background electrolyte and the separations are irreproducible. The presence of the anionic surfactant (SDS) in the buffer is the most probable cause of the observed glucose oxidase instability. SDS has an influence on protein conformation and therewith on the activity of glucose oxidase, depending on its concentration [40]. Therefore, we embarked on another strategy to detect biogenic amines with a sufficient sensitivity essential for their analytics in biological samples.

The modification of the working electrode by CNTs is one of the simplest ways by which the sensitivity of the amperometric detection can be increased. Thus, its applicability to our system has been investigated. CNTs, multi-wall as well as single-wall, have become recently very attractive electrode materials with electrocatalytical properties and have been already successfully implemented for amperometric detection of some catecholamines in CE [25] and in microchip electrophoresis [26,27]. However, only model samples and simple separation systems have so far been applied for amperometric detection using CNT-modified electrode in electrophoresis. Therefore, in the first step, the electrocatalytic capabilities of CNTs in the complex separation system developed for simultaneous separation of catecholamines and their cationic metabolites had to be confirmed using a standard mixture. No significant difference between the multi-wall and single-wall CNTs has been observed with amperometric detection using CNT-modified electrodes (even lower background noise were produced by multi-wall CNTs) [26]. Thus, our experiments were restricted just to multi-wall CNTs.

A common coating procedure [27,33,36] has been adopted for covering the working electrode with the modification of the covering step. Because of the tiny dimensions of the electrode surface, the covering was achieved by repeated immersing of the electrode into the DMF suspension of CNTs instead of using the more common method of dropping the suspension onto the electrode surface.

In the first investigation, it has been observed that the induced amplification of the detection signal depends on the number of immersions of the electrode (Fig. 4). After up to 10 immersions, the detection signal appeared with increased intensity without a damaging effect on the baseline. With further increase in the number of CNT layers, the amplification improved only slightly and the background signal became noisier. Consequently, it was concluded that 10 immersions was a reasonable compromise for the preparation of the electrodes. The exact procedure used for covering the working electrode is described in Section 2. As expected, the amplification also depended on the detection voltage. In the region of kinetically controlled oxidation, the higher detection voltage led to a stronger amplification of the detection signal has been observed, until the detection voltage corresponding to diffusion-controlled oxidation was reached. Therefore, our further experiments were performed at a detection voltage at which the oxidation of all analytes was diffusion-controlled

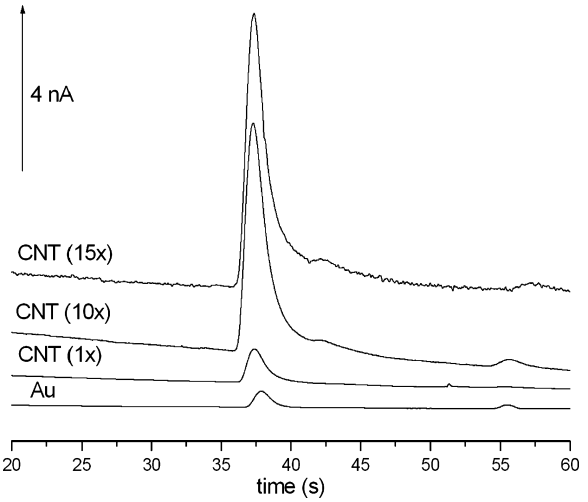


Fig. 4. The detection signal of methoxytyramine (MT) on the microchip using an unmodified Au-electrode and a CNT-modified electrode; the oxidation current is recorded in dependence on the number of CNT layers (see text). Buffer: 20 mM Tris–phosphate buffer (pH 7), separation voltage: 3 kV, injection voltage: 1 kV (3 s), and detection potential 1400 mV.

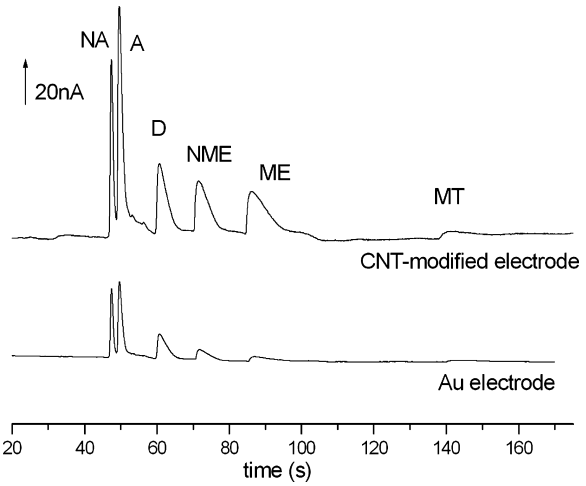


Fig. 5. The electrophoretic separation of selected compounds on the microchip. Buffer: 5 mM borate–phosphate buffer (pH 7) containing 10 mM of SDS and 5 μl/ml of PAMAM dendrimer G1.5, separation voltage: 3 kV, injection voltage: 1 kV (3 s), concentration of analytes: 1 mM, CNT-modified and Au-electrode, and detection potential: 1400 mV.

(1400 mV versus the pseudoreference electrode (microchip) and 1200 mV versus the pseudoreference electrode (CZE)).

The CNT-modified electrode was successfully applied for the amperometric detection of catecholamines and their cationic metabolites after their separation on the microchip (under optimised conditions) with higher detection sensitivity (Fig. 5). The standards were easily detected even at concentrations as low as 10 μM. The LOD for D was calculated to be 1.7 μM, and for A the LOD is 450 nM. These LODs are quite high compared to the values being already achieved by electrophoresis with amperometric detection using different strategies for improving the detection sensitivity or with laser induced fluorescence (LIF) detection. The lowest LODs reported so far for these compounds [9,24] are in lower subnanomolar range and have been obtained using amperometric detection with the help of field-amplified sample stacking [9] or with the application of enzyme catalysed reactions enabling cyclic oxidation of the analytes on the electrode [24]. However, the partition and other complex equilibria employed for the separation has lead to even higher detection limits than are normally obtained with amperometric detection. Nevertheless, we managed to improve the LODs by using of CNT-modified electrode. This improvement made it possible to apply our method for analysis of real samples without any preconcentration or purification, which justifies the applicability of the presented method. The achieved amplification factors (calculated as the ratio between the area of the analyte peak detected by CNT-modified electrode and area of the peak detected by Au electrode) are summarized in Table 1.

The amplification factors of the metabolites are, on average, higher than the amplification factors of catecholamines. This observation is in accordance with the lower coulombic efficiency of the metabolites on the Au electrode described in the literature [24]. The presence of CNTs improves their oxidation behaviour

Table 1
The amplification factors of the amperometric detection signal of studied analytes expressed as the ratio between the area of the analyte peak detected by CNT-modified electrode and area of the peak detected by Au-electrode

Analyte	Amplification factor: A_{CNT}/A_{Au}
NA	2.8
A	3.3
D	3.0
NME	5.5
ME	8.4
MT	2.9

Buffer: 5 mM borate–phosphate buffer (pH 7) containing 10 mM of SDS and 5 μl/ml of PAMAM dendrimer G1.5, separation voltage: 3 kV, injection voltage: 1 kV (3 s), and detection potential: 1400 mV.

and leads to an increase in the oxidation current. Similarly, the CNT-modified electrode also caused amplification of the detection signal in CE. Although lower detection limits were expected with CE, the achieved LODs were similar to those achieved on the microchip due to a higher background noise.

3.3. Analysis of real samples

The biological samples containing catecholamines comprise urine, blood, and brain samples (in the form of cerebrospinal fluid, brain dialysate or brain tissue homogenate). The concentrations of catecholamines and their *O*-methoxylated metabolites in the urine and brain samples are in the low micromolar range, and in the blood samples in the order of nanomoles or even lower. Following from these concentration levels, our experiments were restricted to the brain samples and urine, as in the blood the catecholamines concentration is probably too low for the method described here.

The main objective of the present investigations is to demonstrate the applicability of the new method for the simultaneous determination of all cationic catecholamines and metabolites in the presence of the anionic metabolites. The optimized separation system applicable for short separation lengths and CNT-modified electrode for a sensitive detection were used for the analysis of the brain homogenate and urine samples. All six selected compounds have been detected in mouse brain homogenate as depicted in Fig. 6(a). The identification of the peaks was confirmed by effective ionic mobilities compared with the standards separated in an independent run.

The concentrations of the analytes extracted from the peak areas in the brain electropherogram correspond to a range of nanograms per milligrams of the wet brain tissue, which is in accordance with the typical values of these compounds in the brain homogenate. Other peaks detected in the brain homogenate represent further metabolites originated from catecholamines, which are formed in the following stages of their metabolism. The peaks marked in the electropherogram with the name abbreviation followed by a question mark (and indicated by an arrow) could be ascribed to the metabolite homovanillic acid (HVA), and to another neurotransmitter serotonin (5-HT), respectively, according to our additional experiments with standard mixtures and UV detection.

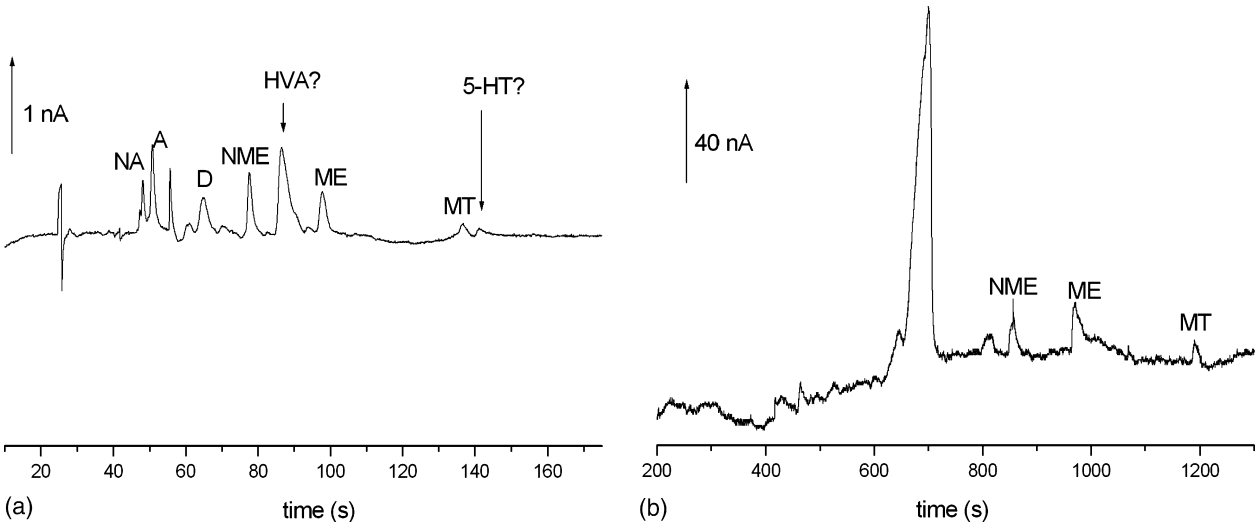


Fig. 6. (a) The analysis of the brain homogenate on the microchip. Buffer: 5 mM borate–phosphate buffer (pH 7) containing 10 mM of SDS and 5 μl/ml of PAMAM dendrimer G1.5, separation voltage: 3 kV, injection voltage: 1 kV (3 s), CNT-modified electrode, detection potential 1400 mV. (b) Urine analysed by CE. Buffer: 5 mM borate–phosphate buffer (pH 7) containing 20 mM of SDS and 5 μl/ml of PAMAM dendrimer G1.5, separation voltage: 20 kV, injection pressure: 250 mbar (6 s), CNT-modified electrode, and detection potential: 1200 mV.

As the high salt concentration in urine distorted the fluid control on the microchip employing the cross-injection, CE was preferred for the urine analysis. The analysis of urine should only demonstrate the applicability of the developed method and, therefore, for sake of simplicity spot urine was used as the sample instead of 24 h urine. The resulting electropherogram of the urine is depicted in Fig. 6(b). High fluctuation in the concentration of the analysed substances in the spot urine sample, however, makes the comparison with the commonly published levels of amines in 24 h urine samples unreasonable. In the investigated urine sample, all three *O*-methoxylated metabolites (marked in the electropherogram) were reliable identified using the effective ionic mobility of the standards. Unlike metabolites, the momentary concentration of the original catecholamines was too low for the successful detection. Typically, the concentration ranges of A and NA and the cationic metabolites are comparable. The peak with the approximate migration time of 700 s probably represents HVA and/or vanillylmandelic acid in large excess. These are the prevailing metabolites present in urine [1]. Even though these acidic metabolites probably co-elute with dopamine at the conditions selected for CE, the determination of adrenaline and noradrenaline is unhindered. Thus, the new method is well suited for diagnosis of pheochromatocytoma, where the monitoring of the levels of A and NA, and/or ME and NME, is required [1].

4. Conclusions

A method for simultaneous separation and sensitive detection of catecholamines and their *O*-methoxylated metabolites has been developed. A novel combination of additives was invented to achieve the complete separation of these compounds even on the short migration length. The application of a second pseudostationary phase by using dendrimers should be particularly emphasized, as the employment of dendrimers in electrophoresis is still in its infancy despite their marked separation potential. Simultaneously, the applicability of CNT-modified electrode in complex separation systems for sensitive amperometric detection of catecholamines and their metabolites is demonstrated. The LODs achieved by the CNT-modified electrode for catecholamines and their cationic metabolites are in micromolar and submicromolar range. For the first time, the application of microchip electrophoresis for the analysis of brain homogenates is presented. The developed method enables the determination of cationic biogenic amines, beside anionic metabolites, within 2.5 min on the microchip device.

Acknowledgements

The authors would like to thank Dr. Albert Enz and Dr. Conrad Gentsch for providing us with the mouse brain samples. Partial funding for this project was provided by Swiss National Science Foundation, Grant numbers 200021-103812/1 and 200020-112190/1.

References

[1] H. Wisser, in: L. Thomas (Ed.), Labor und Diagnose: Indikation und Bewertung von Laborbefunden für Medizinische Diagnostik, TH-Books, Frankfurt, 2000, p. 1062.
[2] J. Bergquist, A. Sciubisz, A. Kaczor, J. Silberring, J. Neurosci. Methods 113 (2002) 1.
[3] J.C. Alvarez, D. Bothua, I. Collignon, C. Advenier, O. Spreux-Varoquaux, Biomed. Chromatogr. 13 (1999) 293.
[4] F.C. Cheng, J.S. Kuo, Y. Shih, J.S. Lai, D.R. Ni, L.G. Chia, J. Chromatogr. 615 (1993) 225.
[5] K.A. Sagar, M.R. Smyth, J. Pharm. Biomed. Anal. 22 (2000) 613.
[6] X.L. Zhu, P.N. Shaw, D.A. Barrett, Anal. Chim. Acta 478 (2003) 259.
[7] T. Yoshitake, J. Kehr, S. Yoshitake, K. Fujino, H. Nohta, M. Yamaguchi, J. Chromatogr. B 807 (2004) 177.

[8] R.P.H. Nikolajsen, A.M. Hansen, Anal. Chim. Acta 449 (2001) 1.
[9] Q.F. Weng, G.W. Xu, K.L. Yuan, P. Tang, J. Chromatogr. B 835 (2006) 55.
[10] H. Siren, U. Karjalainen, J. Chromatogr. A 853 (1999) 527.
[11] K. Vuorensola, H. Siren, U. Karjalainen, J. Chromatogr. B 788 (2003) 277.
[12] T.L. Paxon, P.R. Powell, H.-G. Lee, K.-A. Han, A.G. Ewing, Anal. Chem. 77 (2005) 5349.
[13] S. Tanaka, T. Kaneta, H. Yoshida, Anal. Sci. 6 (1990) 467.
[14] X. Li, W. Jin, Q. Weng, Anal. Chim. Acta 461 (2002) 123.
[15] H. Siren, M. Mielonen, M. Herlevi, J. Chromatogr. A 1032 (2004) 289.
[16] P.J. Ream, S.W. Suljak, A.G. Ewing, K.-A. Han, Anal. Chem. 75 (2003) 3972.
[17] R.H. Zhu, W.T. Kok, Anal. Chem. 69 (1997) 4010.
[18] S.W. Suljak, F.D. Swanek, P.F. Gavin, A.G. Ewing, J. Sep. Sci. 26 (2003) 61.
[19] K. Vuorensola, H. Siren, J. Chromatogr. A 895 (2000) 317.
[20] H.T. Chang, E.S. Yeung, Anal. Chem. 67 (1995) 1079.
[21] M. Du, V. Flanigan, Y.F. Ma, Electrophoresis 25 (2004) 1496.
[22] D. Chen, D. Zhan, C. Cheng, A. Liu, C. Chen, J. Chromatogr. B 750 (2001) 33.
[23] M.A. Schwarz, Electrophoresis 25 (2004) 1916.
[24] M. Vlckova, M.A. Schwarz, Electrophoresis 26 (2005) 2701.
[25] M. Chicharro, A. Sanchez, E. Bermejo, A. Zapardiel, M.D. Rubianes, G.A. Rivas, Anal. Chim. Acta 543 (2005) 84.
[26] J. Wang, G. Chen, M.P. Chatrathi, M. Musameh, Anal. Chem. 76 (2004) 298.
[27] M. Pumera, X. Llopis, A. Merkoci, S. Alegret, Microchim. Acta 152 (2006) 261.
[28] J.A. Lapos, D.P. Manica, A.G. Ewing, Anal. Chem. 74 (2002) 3348.
[29] M.S. Shou, C.R. Ferrario, K.N. Schultz, T.E. Robinson, R.T. Kennedy, Anal. Chem. 78 (2006) 6717.
[30] T. Kappes, P.C. Hauser, Analyst 124 (1999) 1035.
[31] M.A. Schwarz, B. Galliker, K. Fluri, T. Kappes, P.C. Hauser, Analyst 126 (2001) 145.
[32] T. Kappes, B. Galliker, M.A. Schwarz, P.C. Hauser, Trends Anal. Chem. 20 (2001) 133.
[33] W. Zhang, Y. Xie, S. Ai, F. Wan, J. Wang, L. Jin, J. Jin, J. Chromatogr. B 791 (2003) 217.
[34] K. Wu, S. Hu, Microchim. Acta 144 (2004) 131.
[35] J. Wang, Electranalysis 17 (2005) 7.
[36] C. Hu, W. Wang, K. Liao, G. Liu, Y. Wang, J. Phys. Chem. Solids 65 (2004) 1731.
[37] Z.D. Peterson, D.C. Collins, C.R. Bowerbank, M.L. Lee, S.W. Graves, J. Chromatogr. B 776 (2002) 221.
[38] M.A. Schwarz, P.C. Hauser, Anal. Chem. 75 (2003) 4691.
[39] M.A. Schwarz, P.C. Hauser, J. Chromatogr. A 928 (2001) 225.
[40] A.A. Moosavi-Movahedi, J. Iran. Chem. Soc. 2 (2005) 189.

Publication 2

Enzymatic sensitivity enhancement of biogenic monoamines on a chip

Vlčková, M., Schwarz, M.A.

Electrophoresis 2005, 26 (14), 2701-2707

Markéta Vlčková
Maria A. Schwarz

Department of Chemistry,
University of Basel,
Basel, Switzerland

Enzymatic sensitivity enhancement of biogenic monoamines on a chip

Detection of biogenic monoamines in nanomolar concentrations is of great importance for probing the brain chemistry and for their analytics in biological fluids. The sensitivity enhancement of amperometric detection of neurotransmitters (NTs) and their metabolites after their electrophoretic separation on a microchip is presented and is based on coupled enzymatic reactions. The current response of the analyte is amplified by cyclic oxidation on a gold electrode mediated by reduced nicotinamide dinucleotide coenzyme and glucose oxidase enzyme present in the electrophoresis buffer. Using this approach, detection limits of about 10 nM for NTs and their metabolites can be reached.

Keywords: Amperometry / Chip-capillary electrophoresis / Enzyme-catalyzed oxidation / Glucose oxidase / NADH DOI 10.1002/elps.200410396

1 Introduction

The neurotransmitters (NTs) dopamine (D), adrenaline (AD) and noradrenaline (NA) are very important catecholamines (biological monoamines) in the mammalian central nervous system and their main metabolites are methoxytyramine (MT), normetanephrine (NME) and metanephrine (ME). Abnormalities in NTs and thus metabolite concentration levels are important indications of many diseases. Therefore, direct simultaneous determination of these species in the nanomolar range is desirable for the analysis of brain dialysates, urine or blood samples of patients. To date, mostly HPLC methods with electrochemical [1, 2] or fluorescence [3, 4] detection have been employed for the analytics of monoamines in biological samples. Despite low detection limits of these chromatographic methods, long analytical times are often required (up to 60 min). Only relatively few applications of CE have been reported for the separation of catecholamines and related compounds. UV [5–8], fluorescence [9–12], luminescence [13] and also electrochemical [14, 15] detection has been used for these separations but here also, long separation times (about 30 min) are typically required.

Correspondence: Dr. Maria A. Schwarz, Department of Chemistry, University of Basel, Spitalstrasse 51, CH-4056 Basel, Switzerland
E-mail: Maria.Schwarz@unibas.ch
Fax: +41-61-267-1005

Abbreviations: AD, adrenaline; **D**, dopamine; **FAD**, flavine adenine dinucleotide; **G**, glucose; **GOx**, glucose oxidase; **ME**, metanephrine; **MT**, methoxytyramine; **NA**, noradrenaline; **NADH**, nicotinamide dinucleotide reduced form; **NME**, normetanephrine; **NT**, neurotransmitter

In general, a drastic reduction in analysis time can be achieved by employing electrophoretic separation on micromachined separation devices [16]. However, detection sensitivity is one of the few performance parameters that does not directly benefit from system miniaturization, particularly in the case of optical detection systems. Electrochemical detection offers several unique and attractive features for CE of small dimensions including remarkable sensitivity and selectivity. A number of research groups have recently reported the implementation of amperometric detectors on microchip electrophoresis [17, 18]. Microchip-CZE with amperometric detection of NTs in conventional background buffers has been demonstrated and detection limits (LODs) for standard solutions of 10^{-6}–10^{-7} mol/L have been achieved [19]. The lowest LOD reported so far is 1.2×10^{-7} M [20] (decoupling device) and 1.0×10^{-7} M [21] (without decoupler) and was observed for D.

The use of enzyme-catalyzed reactions on the amperometric electrode can influence the oxidation current of NTs in a simple way and is a useful tool for selective and sensitive measurements of biogenic monoamines. Several electrochemical biosensors based on this approach have been described in the literature. Cellobiose dehydrogenase [22], glucose dehydrogenase [23], laccase [24] and tyrosinase [25] are the examples of enzymes being immobilised on an electrode surface in these biosensors. Recently, we have demonstrated that the electrogenerated form of biogenic monoamines, after an electrophoretic separation, reacts with the reduced form of glucose oxidase (GOx) in the presence of glucose (G) [26]. LODs in the range of 10^{-7} M have been reached with the system G/GOx.

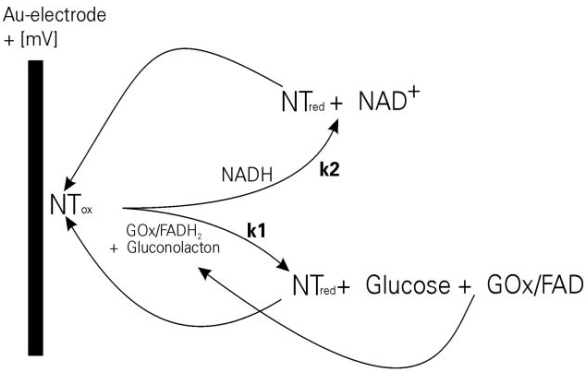


Figure 1. Schematic drawing of cyclic oxidation of the analyte at the gold electrode by two independent enzymatic systems: NADH and G/GOx.

This report describes a new concept of sensitive amperometric detection in microchip electrophoresis of NTs and metabolites using a combined enzyme-catalyzed system consisting of G/GOx and nicotinamide adenine dinucleotide coenzyme in its reduced form (NADH) as shown in Fig. 1. NADH itself can be oxidized at bare electrodes in a two-electron, one-proton reaction; however, high overpotentials (1 V *vs.* Ag/AgCl) [27] are required unless various mediators facilitating its oxidation [28] are used. Our strategy is to use NADH coenzyme together with the G/GOx system as buffer components reducing the electrogenerated (oxidized) form of NTs. This reduction allows their repeated oxidation, which leads to a marked increase in the detection signal.

Up to now, electrocatalytic oxidations of NADH by *o*-quinones derived from 3,4-dihydroxybenzylamine, 4-hydroxy-3-methoxybenzylalcohol [29], 3-hydroxytyramine (D) [30] and 1,2-dihydroxybenzene [31], respectively, have been established. The reaction of NA, AD and the metabolites of NTs with NADH is described here for the first time. The biocatalytic reactions on an amperometric detector that we have investigated provide high selectivity measurements ideally suited for analytics in biological fluids.

2 Materials and methods

2.1 Apparatus and electrodes

The experiments were carried out on a glass microchip purchased from Micralyne (model MC-BF4-TT100, Edmonton, Canada). The separation channel length is 90.28 mm (80.89 mm from intersection) with a semicircular cross section of 50 μm width and 20 μm depth. The intersection is double T-shaped with 100 μm distance between the inlet and outlet part of the injection channel. Pipette tips, serving as reservoirs for buffer and sample, were glued to the top of the chip with an epoxy adhesive (Epo-Tek OG 116, Polyscience, Baar, Switzerland). The chip was mounted on an inverse microscope (model DM IL, Leica, Basel, Switzerland) equipped with an XYZ-micromanipulator holding the working electrode connected to a home-built amperometric detector circuitry, which allows us to set the potential of the working electrode. A two-electrode configuration consisting of an electrophoretic counter electrode and a gold working electrode was employed. The electrophoretic ground electrode was found [32] to provide a sufficiently stable potential and also served as a pseudoreference electrode. A Teflon-coated gold wire (bare wire diameter 75 μm, coated wire diameter 112 μm, Advent Research Materials, England) attached to the micromanipulator was used as a working electrode. The tip of the working electrode was positioned at the endpoint of the separation channel. Injection and separation voltage were produced by two high-voltage power supplies (model CZE1000R, Spellman, Pulborough, England).

2.2 Reagents and methods

The NTs 3-hydroxytyramine hydrochloride (dopamine hydrochloride, D), DL-epinephrine hydrochloride (AD) and DL-norepinephrine hydrochloride (NA) as well as D-(+)-glucose (G) and glucose oxidase (GOx) from *Aspergillus niger* (205 U/mg) and also the buffer constituents Tris and *o*-phosphoric acid 99% were obtained from Fluka (Buchs, Switzerland). The metabolites 3-methoxy-4-hydroxyphenethylamine (3-methoxytyramine, MT), D,L-normethanephrine hydrochloride (NME) and D,L-metanephrine hydrochloride (ME) as well as β-nicotinamide adenine dinucleotide, reduced form (NADH) were supplied by Sigma-Aldrich (Buchs, Switzerland). All reagents were of analytical grade. Deionized water was used to prepare aqueous solutions throughout the study. The buffer was prepared from 10 mM Tris adjusted by phosphoric acid to pH 7. Fresh sample and buffer solutions were prepared daily and filtered through a 0.20 μm filter before introducing the solutions to the chip. The separation channel was preconditioned with a solution of 1 M NADH (1 min) followed by the buffer (3 min) before every series of measurements. Between runs the channel was flushed with the appropriate running buffer (3 min). The separation voltage was set according to the electrophoretic current and was kept constant. Detection potentials varied in the range 1000–1400 mV with respect to the electrophoretic ground electrode.

3 Results and discussion

3.1 Principle of amplification

The oxidation of NTs at a gold electrode initially leads to the corresponding *o*-quinones undergoing further oxidative degradation. Recently, the reduced form of flavine adenine dinucleotide coenzyme (FAD) of the GOx enzyme was demonstrated to react with the quinones on forming original NTs and their metabolites, which are subsequently repeatedly oxidized at the electrode surface (see Fig. 1). The reduced FAD is regenerated by the reaction with G present in the medium on yielding gluconolactone. The cyclic oxidation of the analyte at the electrode causes an amplification of the detection signal and makes it possible to detect the NT and their metabolites in the concentration in order of 10^{-7} M [26].

FAD coenzyme responsible for the cyclic oxidation of NT is tightly bound to the enzymatic protein. Therefore, the presence of a protein in the separation system cannot be avoided. The coupled reaction, which regenerates the reduced form of FAD, is the only way to keep the protein concentration low enough to suppress its pronounced adsorption in the separation channel. It is therefore advantageous to work with a coenzyme capable of independent existence, which can be added in large excess, thus avoiding the need for its regeneration. Nicotinamide dinucleotide is one such coenzyme, and the oxidation of its reduced form (NADH) by D has already been described in the literature [30].

Indeed, the presence of a large excess of NADH in the buffer has led to the cyclic oxidation of all tested NTs and their metabolites at a gold electrode surface, and the detection signal has been amplified similarly as with the G/GOx system. The combination of both amplification systems as demonstrated in Fig. 1 may lead to the further improvement of detection limits.

3.2 Optimization of separation conditions

Obviously, with a higher concentration of NADH in the system, more redox cycles of the analyte can be run and a higher amplification factor can be achieved. If the excess of NADH is sufficiently large, the amplification should be limited only by the transport of the analyte to the electrode, by the kinetics of the reaction and by the time spent at the electrode. However, such a high concentration of NADH, which is an anion at neutral pH, significantly increases the ionic strength of the buffer and thus the electrophoretic current at a constant separation voltage. Such a high electrophoretic current then significantly lowers the detection response at the working electrode unless an amperometric detector with a decoupler is used.

In order to ensure constant detection conditions as well as constant electrophoretic conditions during separation, the measurements were carried out in a constant current mode. A reasonable compromise between the highest possible excess of NADH in BGE and duration of the measurements at constant current mode was achieved with an NADH concentration of 5 mM. The peak area (*A*) of NME (chosen randomly for preliminary experiments) as well as the calculated amplification factor (defined as Amp = A/A_0, in which A_0 and A are the peak areas of NME in the buffer without NADH and with the corresponding concentration of NADH, respectively) increased markedly with the increasing concentration of NADH in the buffer up to 5 mM (see Fig. 2). Further increase might be expected with higher concentrations of NADH. However, the separation would take much longer.

In order to improve the amplification as much as possible (*i.e.,* to reach the lowest possible detection limits) while maintaining a convenient analysis time (not longer than 120 s), a combination of two amplification systems (NADH and G/GOx) was tested instead of further increasing the NADH concentration in the buffer. The addition of previously optimized [26] concentrations of G and GOx (100 mM and 13 μM) to the buffer containing 5 mM of NADH caused further amplification of the detection signal of NME (single point in Fig. 2).

The detection potential of these introductory experiments was set at 1200 mV. However, its influence on the amplification of the signal was also investigated because of the reported G/GOx amplification dependence on the detection potential [26]. The peak area of NME both in the presence and absence of NADH in the running buffer increased with the increasing detection potential. However, the amplification factor fluctuated with no obvious trend (Fig. 2). To cope with such an indistinct observation, the systematic measurements with the NTs and metabolites were carried out at three different detection voltages (1000, 1200 and 1400 mV). The previously reported NADH oxidation [27] at the gold electrode did not take place at these detection potentials. With a 5 mM concentration, no peak for NADH was detected until the detection potential was 1500 mV. Note that our detection potentials are measured relative to the pseudoreference electrophoretic ground electrode and are shifted to higher values in comparison to the potentials measured *versus* standard Ag/AgCl electrode [32].

The pH of the BGE used for our measurements (pH 7, 10 mM Tris-phosphate buffer) was selected as a compromise with regard to the optimum pH for NADH oxidation (pH 6.8) [33] and to the pH used in measurements with G/GOx amplification (pH 7.2) [26]. All substances that were tested were separated successively in the buffer without

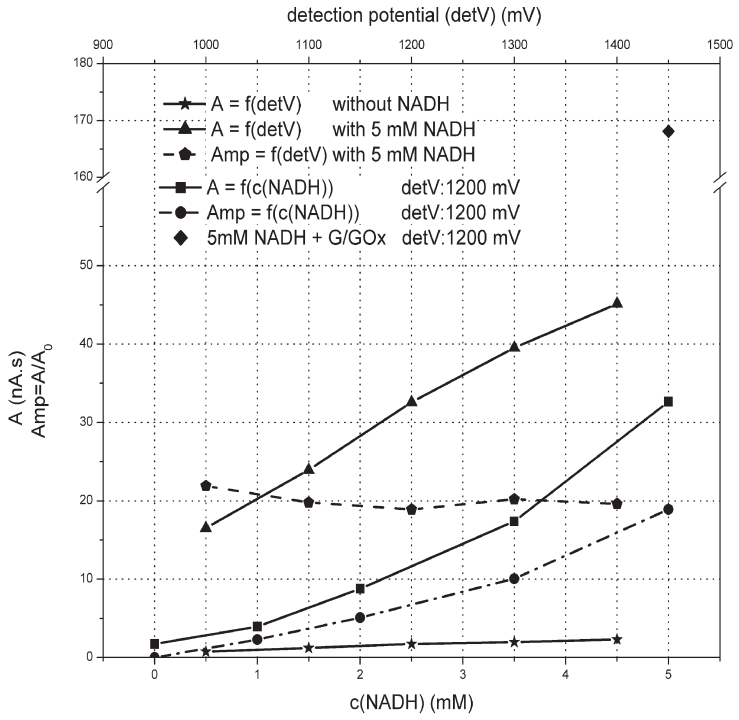


Figure 2. Peak areas (*A*) and amplification factors (Amp) of NME in dependence on concentration of NADH in the running buffer (at a detection potential of 1200 mV) and on the detection potential (Det *V*) (at a constant NADH concentration of 5 mM) and peak area of NME in the presence of combined NADH + G/GOx amplification system. Buffer, 10 mM Tris-phosphate; 100 mM G, 13 μM GOx, 5 mM NADH; electrophoretic current, 1.47 μA; injection voltage, 1 kV (3 s); Au electrode.

any additive, in the buffer with addition of the system G/GOx (100 mM/13 μM), then in the buffer with addition of 5 mM NADH and finally in the buffer containing both amplification systems. The comparison of the electropherograms of ME in all of these separation systems at detection potential 1200 mV is shown in Fig. 3.

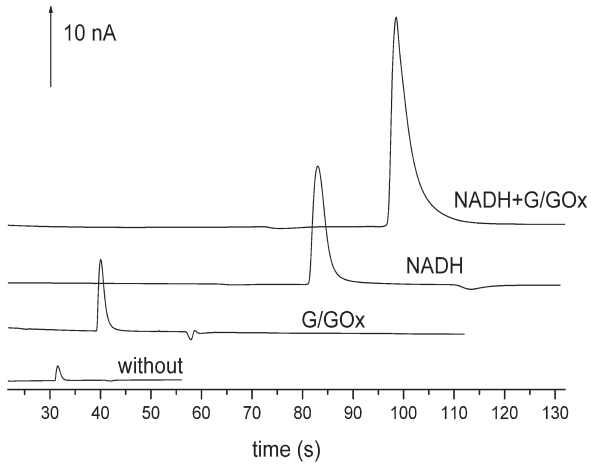


Figure 3. Electropherogram of ME in the buffer without any additive, in the presence of G/GOx, NADH and combined NADH + G/GOx amplification system. Buffer, 10 mM Tris-phosphate; 100 mM G, 13 μM GOx, 5 mM NADH; electrophoretic current, 1.47 μA; injection voltage, 1 kV (3 s); Au electrode; detection potential, 1200 mV.

Every measurement with a given analyte with the respective composition of the BGE and with the respective detection voltage was carried out three times and the mean value of peak areas was recorded. One randomly chosen analyte (NME) was examined in more detail with respect to the reproducibility of the measurements with amplification of the detection signal. Seven subsequent measurements in buffer containing NADH yielded a constant area with the accuracy 90 ± 10%; similar accuracy was also observed in the buffer with system G/GOx and buffer containing both of the systems. The peak areas (*A*) of all measured NTs and their metabolites and the calculated amplification factor (Amp = A/A_0) are summarized in Table 1.

3.3 The multiple enzyme system NADH/GOx

The amplification factors obtained with metabolites of NTs are considerably higher with all of the studied amplification systems in comparison to the amplification of NTs. This finding is in accord with the previously published amplification with system G/GOx [26] even though the amplification by system G/GOx is generally lower in our measurements probably due to different experimental conditions (separation voltage and thus the detection voltage [32]). The differences in the amplification of metabolites and of NTs were ascribed to the different

Table 1. Amplification factors (Amp.) of NTs and their metabolites with system G/GOx, NADH and combined enzyme system (NADH/GOx) at different detection potentials (Det *V* – detection voltage)

Analyte	Det *V* (mV)	Amp. G/GOx	Amp. NADH	Amp. NADH/GOx
D	1 000	6.8*x*	4.7*x*	17.2*x*
	1 200	3.6*x*	5*x*	14.2*x*
	1 400	–	7.1*x*	–
NA	1 000	4.1*x*	8.3*x*	25.3*x*
	1 200	3.1*x*	6*x*	21.2*x*
	1 400	–	5.7*x*	–
AD	1 000	1.6*x*	4.5*x*	12.2*x*
	1 200	1.5*x*	2.5*x*	8.5*x*
	1 400	–	3.4*x*	–
MT	1 000	84.4*x*	54.3*x*	403.1*x*
	1 200	25.9*x*	28.2*x*	182*x*
	1 400	–	20.2*x*	–
NME	1 000	36.9*x*	21.9*x*	212.9*x*
	1 200	11.1*x*	18.9*x*	74.7*x*
	1 400	–	19.6*x*	–
ME	1 000	17.6*x*	19.9*x*	64.2*x*
	1 200	11.7*x*	25.2*x*	66.6*x*
	1 400	–	9.5*x*	–

Buffer, 10 mM Tris-phosphate; 100 mM G, 13 μM GOx, 5 mM NADH; electrophoretic current, 1.47 nA; injection voltage, 1 kV (3 s); Au electrode

stability of quinone derivatives and also to the kinetic aspects [26]. Another explanation may be the lower coulombic efficiency of the metabolites. The term coulombic efficiency describes the readiness of a substance to undergo the electrode reaction and may be calculated as follows. The theoretical charge passing through the amperometric detector is given by Eq. (1).

$$Q_{th} = nFz \quad (1)$$

In Eq. (1), *n* is the number of moles of the sample calculated from the concentration of the analyte and from the injected volume (given by the dimensions of cross section of the chip), *F* is the Faraday constant and *z* is the number of electrons involved in the oxidation process. The real charge (Q_r) passed through the detector is then the calculated peak area given in the units nA × s. The coulombic efficiency is the fraction of Q_r/Q_{th} and is often given as a percentage value.

As was expected, the coulombic efficiencies of NTs, without enzyme systems in the CE buffer, and of their metabolites, calculated from our measurements, differ significantly from each other. While the coulombic efficiencies of D are approximately 100%, the coulombic efficiencies of metabolites lie between 2 and 40% depending on the actual metabolite and on the detection potential. After addition of the amplification systems, the coulombic efficiency increased to over 100% in the case of both NTs and metabolites because the analyte is regenerated by cyclic oxidation and is oxidized repeatedly.

In all cases, the extent of amplification of the peak area of the respective compound by system G/GOx and by NADH (see Table 1) is similar. It suggests that the rate constants of both reactions are of the same order for all of the compounds we have studied. This suggestion is supported by the comparable values of the rate constant for the reaction between NADH and benzoquinone (2.9×10^6/M × s) [31] and between GOx and the oxidised form of D (1.2×10^6/M × s) [34] available in the literature. The oxidized form of D and benzoquinone can be compared regarding the rate constant [35].

As it is shown in Fig. 2, the dependence of the amplification factor on the detection potential was not proved for NADH (the differences may be ascribed to the errors of measurements) contrary to the system G/GOx and hence to the combined enzyme systems. However, at high detection voltages (1400 mV), at which the absolute values of the peak areas are maximal, only NADH amplified the detection signal. G/GOx present in the background electrolyte caused a large baseline noise indicating its oxidation on the electrode at this potential.

The question of a possible mutual interaction of the NADH and G/GOx systems when used simultaneously may be investigated by a direct comparison of the amplification factors obtained with system G/GOx, with NADH and with the combination of both of these systems. Generally, the catalytic effects might be additive, synergistic or inhibitive. If the actions are independent (additive catalytic effects) the amplification factor of a combined system NADH + G/GOx should be a multiple of the amplification factors of NADH and G/GOx. With synergism or inhibition the resulting amplification is then markedly higher or lower. Following from the amplification factors of NTs (AD, NA, D) from Table 1, the amplification reactions are highly likely to be parallel and competitive. Their simultaneous presence in the buffer therefore causes neither the enhancement nor suppression of the effects; both systems act independently. Markedly lower than multiple amplification factors obtained with the metabolites could be explained by the limited time spent at the electrode preventing the occurrence of more redox cycles. This means that the maximum achievable peak areas (their absolute values), and hence the extent of amplification, are determined not only by the kinetics of the reactions participating on the amplification, but

also by the velocity of the analyte during passing the detector and by the dispersion of the zone. These parameters determine the time spent by the analyte at the detector, which is decisive in determining the number of redox cycles, and the time is given by the mobility of the analyte and by the EOF. In the case of our independent multiple enzyme systems NADH and G/GOx, the amplification is thus a function of the rate constants of both parallel reactions, the mobility of the analyte and the mobility of the EOF, mathematically expressed as $Amp = f(k_1, k_2, \mu, \mu_{EOF})$.

The LOD achieved with a combination of NADH with G/GOx has been determined for methoxytyramine, which yields the highest amplification factor (Table 1). The lowest detected concentration of MT with the combined enzyme system was 47 nM (Fig. 4) and LOD calculated from an S/N of 3/1 is even lower and is equal to 12 nM (absolute amount of analyte: 0.97 fmol). For a comparison, the LOD of MT in the same buffer without any additive calculated from S/N = 3 has been determined to be 80 μM. Thus, the application of combined enzyme system causes a drastic fall in LOD through more than three orders of magnitude. The LODs in the low nanomolar range are expected for all of the studied compounds because the absolute peak areas obtained are well comparable. This is to our knowledge the lowest LOD for NTs published yet. The detector response (at the detection potential 1200 mV) was linear in the whole studied range from 50 nM to 50 μM ($A = 0.45 \times c + 1.09$, $R^2 = 0.9952$).

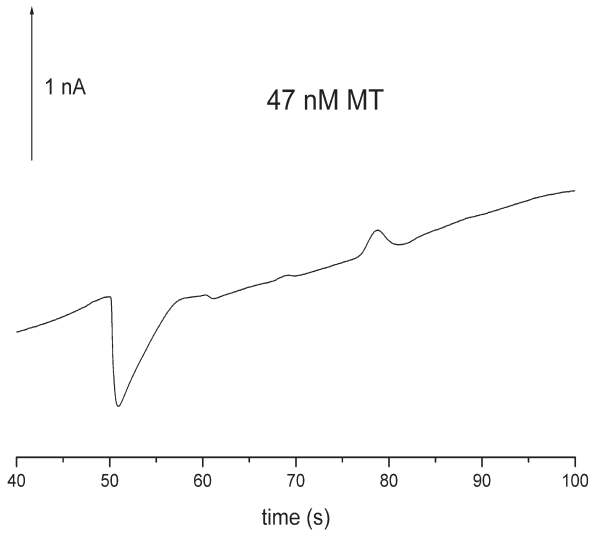


Figure 4. Electropherogram of MT (47 nM) in the presence of combined NADH + G/GOx amplification system. Buffer, 10 mM Tris-phosphate, 100 mM G, 13 μM GOx, 5 mM NADH, electrophoretic current 1.47 μA, injection voltage 1 kV (3 s), Au electrode, detection potential 1200 mV.

Addition of Ca^{2+} or Mg^{2+} ions (which are reported in [36] to enhance the catalytic NADH oxidation) to the BGE containing NADH was also examined. However, no increased amplification was observed. The observed peak was even smaller. These ions probably facilitate the oxidation of NADH at the electrode and in this way decrease the amount of NADH available for reaction with the oxidised analyte.

4 Concluding remarks

It has been demonstrated that NTs and their metabolites could be detected in a nanomolar concentration after their electrophoretic analysis on a microchip equipped with an amperometric detector in the presence of two enzymatic systems in the background electrolyte, NADH and G/GOx. According to our results, the systems act independently and amplify the detector response of the compounds by regeneration of the analytes after their oxidation at the electrode. The rates of both reactions are comparable and amplification is reproducible. The method described in this paper is therefore well suited for the selective and sensitive determination of NTs and their metabolites in clinical samples, such as urine or plasma. The oxidation of NADH by AD, NA and all of the metabolites involved in the study is described herein for the first time. In particular, it should be highlighted that the LOD of metabolites are in the range of the good oxidizable molecules D, AD and NA. This is an important fact for the monitoring of the concentration of certain species in patients according to the metabolic pathway of monoamines in the human brain [37]. The application of this coupled enzymatic system is suitable for any separation procedure coupled with amperometric detection.

This work was supported by the Swiss National Science Foundation, Grant number 200021-103812/1.

Received December 14, 2004

5 References

[1] Sarre, S., Michotte, Y., Herregodts, P., Deleu, D., Klippel, N. D., Ebinger, G., *J. Chromatogr.* 1992, *575*, 207–212.

[2] Sagar, K. A., Smyth, M. R., *J. Pharm. Biomed. Anal.* 2000, *22*, 613–624.

[3] Zhu, X., Shaw, P. N., Barrett, D. A., *Anal. Chim. Acta* 2003, *478*, 259–269.

[4] Fotopoulou, M. A., Ioannou, P. C., *Anal. Chim. Acta* 2002, *462*, 179–185.

[5] Kartsova, L. A., Sidorova, A. A., Kazakov, V. A., Bessonova, E. A., Yashin, A. Y., *J. Anal. Chem.* 2004, *59*, 737–741.

[6] Siren, H., Karjalainen, U., *J. Chromatogr. A* 1999, *853*, 527–533.

[7] Garcia, A., Heinaenen, M., Jimenez, L. M., Barbas, C., *J. Chromatogr. A* 2000, *871*, 341–350.

[8] Siren, H., Mielonen, M., Herlevi, M., *J. Chromatogr. A* 2004, *1032*, 289–297.

[9] Park, Y., Zhang, X., Rubakhin, S., Sweedler, J., *Anal. Chem.* 1999, *71*, 4997–5002.

[10] Chang, H. T., Yeung, E. S., *Anal. Chem.* 1995, *67*, 1079–1083.

[11] Du, M., Flanigan, V., Ma, Y., *Electrophoresis* 2004, *25*, 1496–1502.

[12] Parrot, S., Sauvinet, V., Riban, V., Depaulis, A., Renaud, B., Denoroy, L., *J. Neurosci. Methods* 2004, *140*, 29–38.

[13] Zhu, R., Kok, W. T., *Anal. Chem.* 1997, *69*, 4010–4016.

[14] Zhang, L., Qv, S., Wang, Z., Cheng, J., *J. Chromatogr. B* 2003, *792*, 381–385.

[15] Li, X., Jin, W., Weng, Q., *Anal. Chim. Acta* 2002, *461*, 123–130.

[16] Schwarz, M. A., Hauser, P. C., *Anal. Chem.* 2003, *75*, 4691–4695.

[17] Lacher, N. A., Garrison, K. E., Martin, R. S., Lunte, S. M., *Electrophoresis* 2001, *22*, 2526–2536.

[18] Vandaveer, W. R., Pasas, S. A., Martin, R. S., Lunte, S. M., *Electrophoresis* 2002, *23*, 3667–3677.

[19] Schwarz, M. A., Hauser, P. C., *J. Chromatogr. A* 2001, *928*, 225–232.

[20] Wu, C.-C., Wu, R.-G., Huang, J.-G., Chang, H.-C., *Anal. Chem.* 2003, *75*, 947–952.

[21] Liu, Y., Vickers, J. A., Henry, C. S., *Anal. Chem.* 2004, *76*, 1513–1517.

[22] Stoica, L., Lindgren-Sjolander, A., Ruzgas, T., Gorton, L., *Anal. Chem.* 2004, *76*, 4690–4696.

[23] Lisdat, F., Wollenberger, U., Makower, A., Hortnagl, H., Pfeiffer, D., Scheller, F. W., *Biosens. Bioelectron.* 1997, *12*, 1199–1211.

[24] Leite, O. D., Fatibello, O., Barbosa, A. D., *J. Braz. Chem. Soc.* 2003, *14*, 297–303.

[25] Yao, T., Ho, C. O., *Bunseki Kagaku* 2002, *51*, 469–472.

[26] Schwarz, M. A., *Electrophoresis* 2004, *25*, 1916–1922.

[27] Pariente, F., Tobalina, F., Moreno, G., Hernandez, L., Lorenzo, E., Abruna, H. D., *Anal. Chem.* 1997, *69*, 4065–4075.

[28] Prieto-Simon, B., Fabregas, E., *Biosens. Bioelectron.* 2004, *19*, 1131–1138.

[29] Ueda, C., Tse, D. C.-S., Kuwana, T., *Anal. Chem.* 1982, *54*, 850–856.

[30] Tse, D. C.-S., Kuwana, T., *Anal. Chem.* 1978, *50*, 1315–1318.

[31] Murthy, A. S. N., Sharma, J., *Talanta* 1998, *45*, 951–956.

[32] Schwarz, M. A., Galliker, B., Fluri, K., Kappes, T., Hauser, P. C., *Analyst* 2001, *126*, 145–151.

[33] Chen, J., Bao, J., Cai, C., Lu, T., *Anal. Chim. Acta* 2004, *516*, 29–34.

[34] Battaglini, F., Koutroumanis, M., English, A. M., Mikkelsen, S. R., *Bioconjug. Chem.* 1994, *5*, 430–435.

[35] Mizutani, F., Yabuki, S., Asai, M., *Biosens. Bioelectron.* 1991, *6*, 305–310.

[36] Mano, N., Kuhn, A., Menu, S., Dufourc, E. J., *Phys. Chem. Chem. Phys.* 2003, *5*, 2082–2088.

[37] Maruyama, W., Naoi, M., Narabayashi, H., *J. Neurolog. Sci.* 1996, *139*, 141–148.

Publication 3

Microchip Affinity Capillary Electrophoresis Applications and Recent Advances

Vlčková, M., Stettler, A., Schwarz, M.A.

J. Liq. Chromatogr. Relat. Technol. 2006, 29 (7-8), 1047-1076

Journal of Liquid Chromatography & Related Technologies®, 29: 1047–1076, 2006

ISSN 1082-6076 print/1520-572X online
DOI: 10.1080/10826070600574754

Microchip Affinity Capillary Electrophoresis: Applications and Recent Advances

Markéta Vlčková, Alexandra R. Stettler, and Maria A. Schwarz
Universität Basel, Departement Chemie, Basel, Switzerland

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

Address correspondence to Maria A. Schwarz, Universität Basel, Departement Chemie, Spitalstrasse 51, CH-4056 Basel, Switzerland. E-mail: maria.schwarz@unibas.ch

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

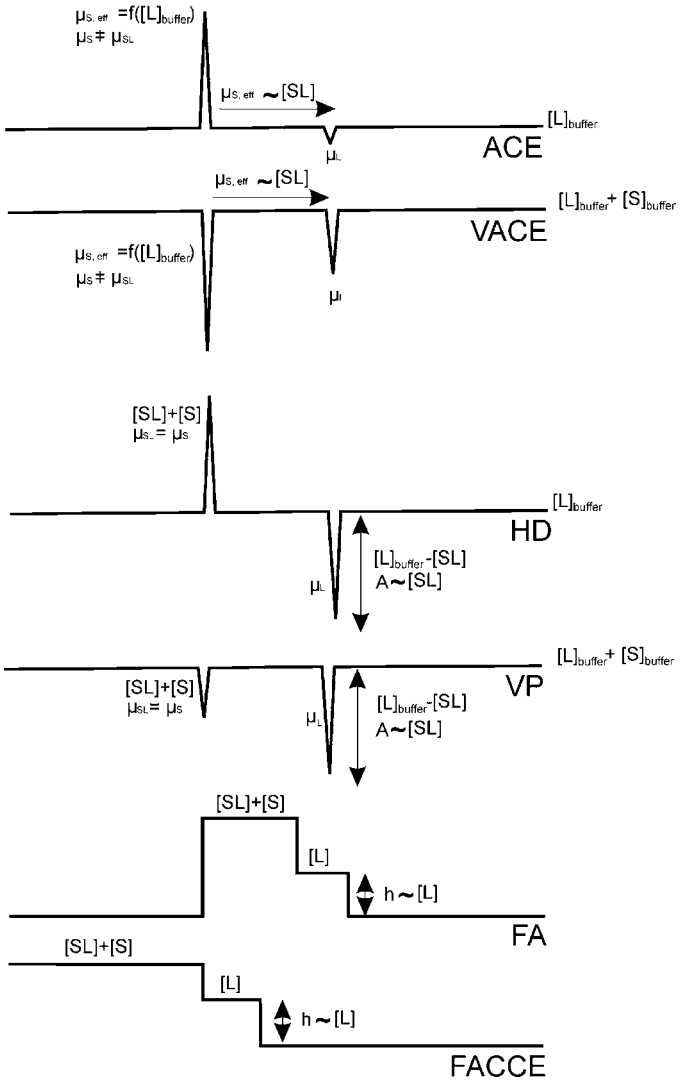


Figure 1. Schematic elution profiles of ACE, VACE, HD, VP, FA and FACCE for the reaction: A – area of detection signal; μ_{eff} – effective mobility. The arrows indicate the parameter $S + L \rightleftharpoons SL$ measured in dependence on the concentration of free ligand, $[L]_{buffer}$, in the background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

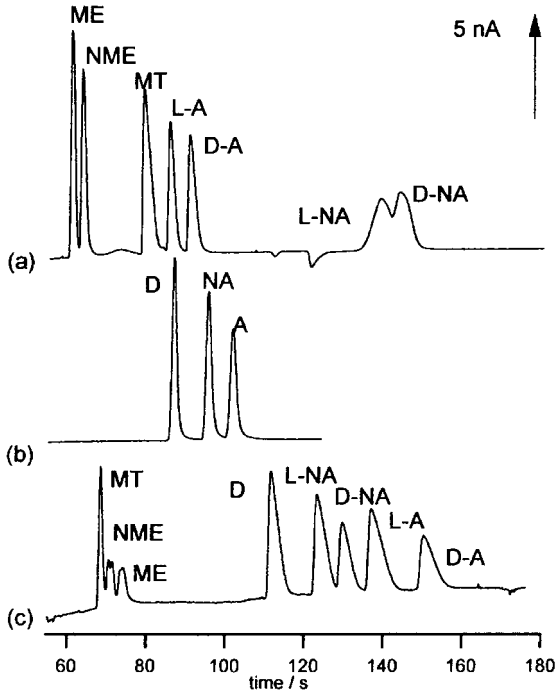


Figure 2. Electropherogram for noradrenaline (NA) and adrenaline (A) in the presence of dopamine (D), methoxytyramine (MT), normetanephrine (NME), and metanephrine (ME). Conditions: buffers, (a) 10 mM TRIS, pH 3.0, 10 mg/mL CMCD, 38 mmol/L 18-crown-6; (b) 20 mM MES, pH 6.0; 5 mg/mL dendrimer; (c) 20 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. From [32] with permission.

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

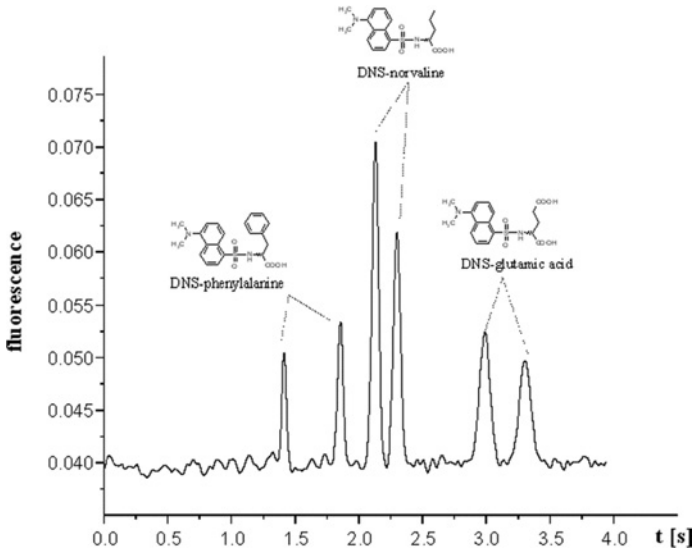


Figure 3. Chiral separation of three compounds in less than 3.5 s. conditions: electrolyte: 2% HS-γ-CD, 25 mM triethylammonium phosphate buffer, pH 2.5. From [35] with permission. Conditions see Table 1.

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

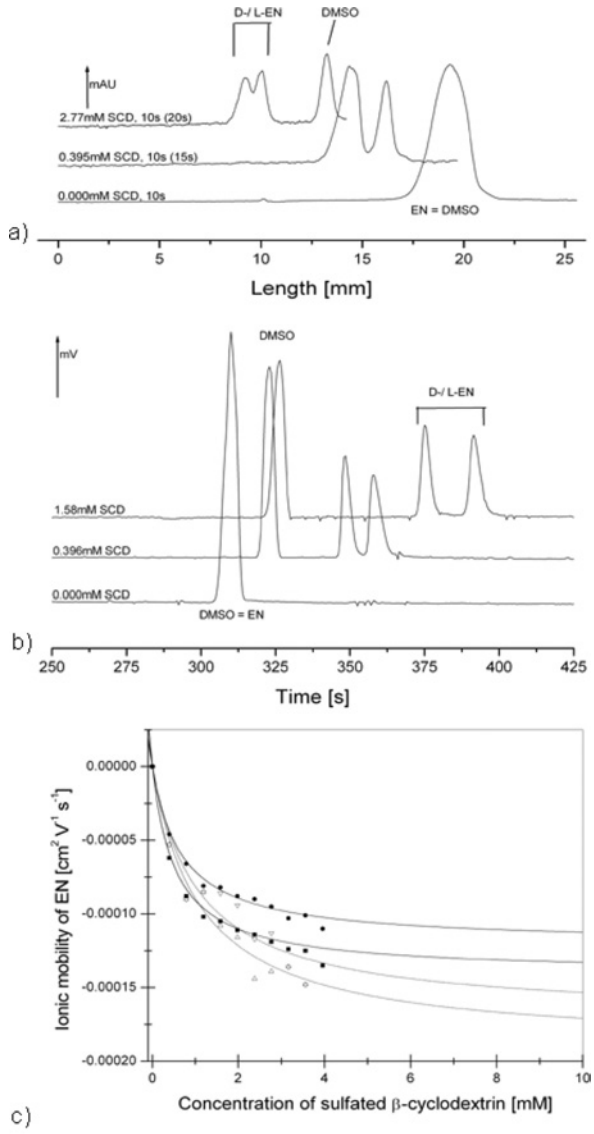


Figure 4. Electropherograms measured on microchip (a) and capillary (b). Buffer: 10 mM TRIS at 210 nm containing sulfated cyclodextrin (sCD); internal standard: DMSO; microchip: 2 mM epinephrine (EN) at pH 7.37. The separation times differed from 10 s up to 20 s (converted to 10 s) at 280 V/cm using 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.

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.

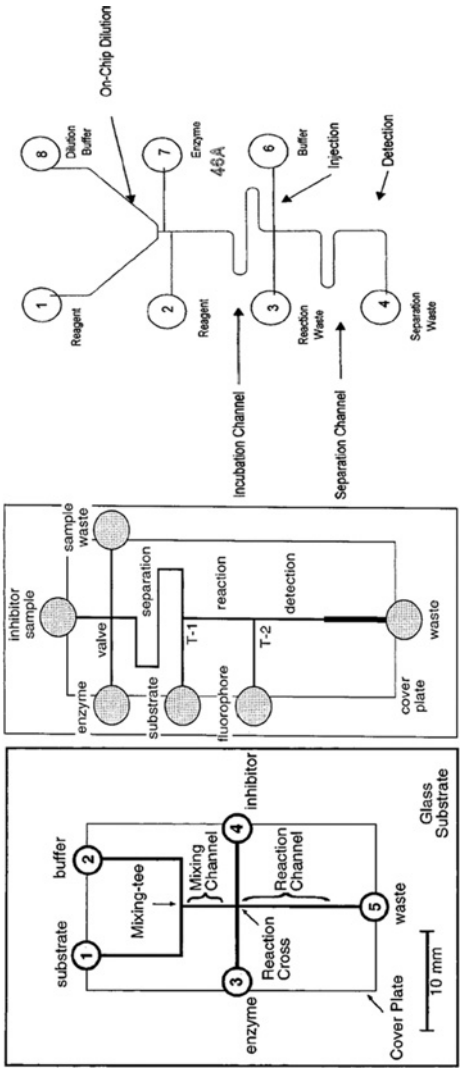


Figure 5. Schematic of the chips used in [51, 52, 56]. With the chip on the left side, dilution of the substrate is possible. Enzyme and a possible inhibitor are added simultaneously. On the chip in the middle, the enzyme first interacts with the inhibitor before coming to the substrate, the product 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. From [51, 52, 55] with permission.

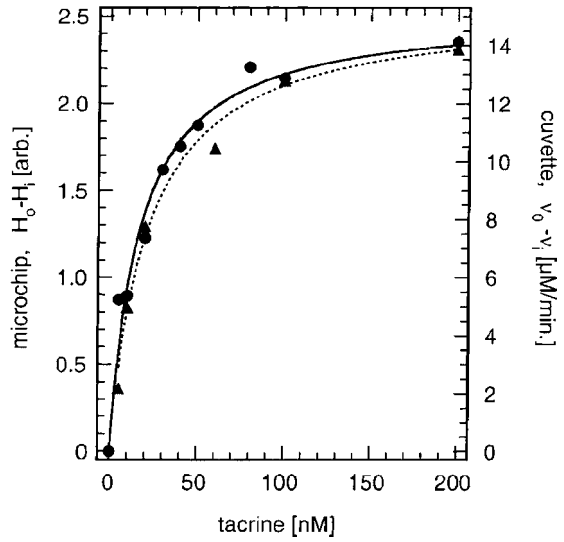


Figure 6. Difference in initial and inhibited enzyme reaction rates versus tacrine concentration for microchip- and cuvette-based assays. Left axis shows values for difference in peak height ($H_0 - H_i$) for the microchip assay (▲); right axis, the difference in initial reaction rates ($v_0 - v_i$) 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

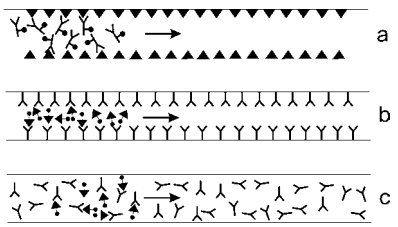


Figure 7. Schema of immunoassays on microfluidic devices.

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

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]

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

REFERENCES

1. Guijt, R.M.; Frank, J.; van Dedem, G.W.K.; Baltussen, E. Recent advances in affinity capillary electrophoresis. Electrophoresis **2000**, *21*, 3905–3918.
2. Busch, M.H.A.; Carels, L.B.; Boelens, H.F.M.; Kraak, J.C.; Poppe, H. Comparison of five methods for the study of drug-protein binding in affinity capillary electrophoresis. J. Chromatogr. A **1997**, *777*, 311–328.
3. Tanaka, Y.; Terabe, S. Estimation of binding constants by capillary electrophoresis. J. Chromatogr. B **2002**, *768*, 81–92.
4. Stettler, A.R.; Schwarz, M.A. Affinity capillary electrophoresis on microchips. J. Chromatogr. A **2005**, *1063*, 217–225.
5. Busch, M.H.A.; Boelens, H.F.M.; Kraak, J.C.; Poppe, H. Vacancy affinity capillary electrophoresis, a new method for measuring association constants. J. Chromatogr. A **1997**, *775*, 313–326.
6. Rundlett, K.L.; Armstrong, D.W. Methods for the determination of binding constants by capillary electrophoresis. Electrophoresis **2001**, *22*, 1419–1427.
7. Busch, M.H.A.; Kraak, J.C.; Poppe, H. Principles and limitations of methods available for the determination of binding constants with affinity capillary electrophoresis. J. Chromatogr. A **1997**, *777*, 329–353.
8. Alarie, J.P.; Jacobson, S.C.; Ramsey, J.M. Electrophoretic injection bias in a microchip valving scheme. Electrophoresis **2001**, *22*, 312–317.
9. Ermakov, S.V.; Jacobson, S.C.; Ramsey, J.M. Computer simulations of electrokinetic injection techniques in microfluidic devices. Anal. Chem. **2000**, *72*, 3512–3517.
10. Zhang, C.-X.; Manz, A. Narrow sample channel injectors for capillary electrophoresis on microchips. Anal. Chem. **2001**, *73*, 2656–2662.
11. Lee, N.Y.; Yamada, M.; Seki, M. Pressure-driven sample injection with quantitative liquid dispensing for on-chip electrophoresis. Anal. Sci. **2004**, *20*, 483–487.
12. Solignac, D.; Gijs, M.A.M. Pressure pulse injection: a powerful alternative to electrokinetic sample loading in electrophoresis microchips. Anal. Chem. **2003**, *75*, 1652–1657.

13. Ostergaard, J.; Heegard, N.H.H. Capillary electrophoresis frontal analysis: Principles and applications for the study of drug-plasma protein binding-Electrophoresis **2003**, *24*, 2903–2913.
14. Gao, J.Y.; Dubin, P.L.; Muhoberac, B.B. Measurement of the binding of proteins to polyelectrolytes by frontal analysis continuous capillary electrophoresis. Anal. Chem. **1997**, *69*, 2945–2951.
15. Backhouse, C.J.; Crabtree, H.J.; Glerum, D.M. Frontal analysis on a microchip. Analyst **2002**, *127*, 1169–1175.
16. Kutter, J.P.; Jacobson, S.C.; Ramsey, J.M. Integrated microchip device with electrokinetically controlled solvent mixing for isocratic and gradient elution in micellar electrokinetic chromatography. Anal. Chem. **1997**, *69*, 5165–5171.
17. Moore, A.W.; Jacobson, S.C.; Ramsey, J.M. Microchip separations of neutral species via micellar electrokinetic capillary chromatography. Anal. Chem. **1995**, *67*, 4184–4189.
18. vonHeeren, F.; Verpoorte, E.; Manz, A.; Thormann, W. Micellar electrokinetic chromatography separations and analyses of biological samples on a cyclic planar microstructure. Anal. Chem. **1996**, *68*, 2044–2053.
19. Rodriguez, I.; Lee, H.K.; Li, S.F.Y. Microchannel electrophoretic separation of biogenic amines by micellar electrokinetic chromatography. Electrophoresis **1999**, *20*, 118–126.
20. Culbertson, C.T.; Jacobson, S.C.; Ramsey, J.M. Microchip devices for high-efficiency separations. Anal. Chem. **2000**, *72*, 5814–5819.
21. Ro, K.W.; Lim, K.; Kim, H.; Hahn, J.H. Poly(dimethylsiloxane) microchip for precolumn reaction and micellar electrokinetic chromatography of biogenic amines. Electrophoresis **2002**, *23*, 1129–1137.
22. Wallenborg, S.R.; Bailey, C.G. Separation and detection of explosives on a microchip using micellar electrokinetic chromatography and indirect laser-induced fluorescence. Anal. Chem. **2000**, *72*, 1872–1878.
23. Suljak, S.W.; Thompson, L.A.; Ewing, A.G. Improving resolution for channel-format chip-based electrophoresis with electrochemical array detection J. Sepn. Sci. **2004**, *27*, 13–20.
24. Garcia, C.D.; Dressen, B.M.; Henderson, A.; Henry, C.S. Comparison of surfactants for dynamic surface modification of poly(dimethylsiloxane) microchips. Electrophoresis **2005**, *26*, 703–709.
25. Sera, Y.; Matsubara, N.; Otsuka, K.; Terabe, S. Sweeping on a microchip: Concentration profiles of the focused zone in micellar electrokinetic chromatography. Electrophoresis **2001**, *22*, 3509–3513.
26. Vespalec, R.; Bocek, P. Calculation of stability constants for the chiral selector-enantiomer interactions from electrophoretic mobilities. J. Chromatogr. A **2000**, *875*, 431–445.
27. Belder, D.; Ludwig, M. Microchip electrophoresis for chiral separations. Electrophoresis **2003**, *24*, 2422–2430.
28. Il Cho, S.; Shim, J.; Kim, M.S.; Kim, Y.K.; Chung, D.S. On-line sample cleanup and chiral separation of gemifloxacin in a urinary solution using chiral crown ether as a chiral selector in microchip electrophoresis. J. Chromatogr. A **2004**, *1055*, 241–245.
29. Ludwig, M.; Kohler, F.; Belder, D. High-speed chiral separations on microchip with UV-detection. Electrophoresis **2003**, *24*, 3233–3238.
30. Kitagawa, F.; Aizawa, S.; Otsuka, K. Rapid enantioseparation of 1-aminoindan by microchip electrophoresis with linear-imaging UV detection. Anal. Sci. **2005**, *21*, 61–65.

31. Olvecka, E.; Masar, M.; Kaniansky, D.; Johnck, M.; Stanislawski, B. Isotachophoresis separations of enantiomers on a planar chip with coupled separation channels. Electrophoresis **2001**, *22*, 3347–3353.
32. Schwarz, M.A.; Hauser, P.C. Chiral on-chip separations of neurotransmitters. Anal. Chem. **2003**, *75*, 4691–4695.
33. Ludwig, M.; Belder, D. Coated microfluidic devices for improved chiral separations in microchip electrophoresis. Electrophoresis **2003**, *24*, 2481–2486.
34. Hutt, L.D.; Glavin, D.P.; Bada, J.L.; Mathies, R.A. Microfabricated capillary electrophoresis amino acid chirality analyser for extraterrestrial exploration. Anal. Chem. **1999**, *71*, 4000–4006.
35. Piehl, N.; Ludwig, M.; Belder, D. Subsecond chiral separations on a microchip. Electrophoresis **2004**, *25*, 3848–3852.
36. Rodriguez, I.; Jin, L.J.; Li, S.F.Y. High-speed chiral separations on microchip electrophoresis devices. Electrophoresis **2000**, *21*, 211–219.
37. Wallenborg, S.R.; Lurie, I.S.; Arnold, D.W.; Bailey, C.G. On-chip chiral and achiral separation of amphetamine and related compounds labeled with 4-fluoro-7-nitrobenzofurazene. Electrophoresis **2000**, *21*, 3257–3263.
38. Schwarz, M.A.; Hauser, P.C. Rapid chiral on-chip separation with simplified amperometric detection. J. Chromatgr. A **2001**, *928*, 225–232.
39. Belder, D.; Deege, A.; Maass, M.; Ludwig, M. Design and performance of microchip electrophoresis instrument with sensitive variable-wavelength fluorescence detection. Electrophoresis **2002**, *23*, 2355–2361.
40. Il Cho, S.; Lee, K.N.; Kim, Y.K.; Jang, J.H.; Chung, D.S. Chiral separation of gemifloxacin in sodium-containing media using chiral crown ether as a chiral selector by capillary and microchip electrophoresis. Electrophoresis **2002**, *23*, 972–977.
41. Skelley, A.M.; Mathies, R.A. Chiral separation of fluorescamine-labeled amino acids using microfabricated capillary electrophoresis devices for extraterrestrial exploration. J. Chromatogr. A **2003**, *1021*, 191–199.
42. Stachowiak, T.B.; Svec, F.; Fréchet, J.M.J. Chip electrochromatography. J. Chromatogr. A **2004**, *1044*, 97–111.
43. Guihen, E.; Glennon, J.D. Recent highlights in stationary phase design for open-tubular capillary electrochromatography. J. Chromatogr. A **2004**, *1044*, 67–81.
44. Svec, F. Recent developments in the field of monolithic stationary phases for capillary electrochromatography. J. Sepn. Sci. **2005**, *28*, 729–745.
45. Peterson, D. Solid supports for micro analytical systems. Lab On A Chip **2005**, *5*, 132–139.
46. He, B. Fabrication of nanocolumns for liquid chromatography. Anal. Chem. **1998**, *70*, 3790–3797.
47. Schweitz, L. Approaches to molecular imprinting based selectivity in capillary electrochromatography. Electrophoresis **2001**, *22*, 4053–4063.
48. Pumera, M. Microchip-based electrochromatography: designs and applications. Talanta **2005**, *66*, 1048–1062.
49. Guijt, R.M.; Baltussen, E.; van Demen, G.W.K. Use of bioaffinity interactions in electrokinetically controlled assays on microfabricated devices. Electrophoresis **2002**, *23*, 823–825.
50. Novakova, S.; Van Dyck, S.; Van Schepdael, A.; Hoogmartens, J.; Glatz, Z. Electrophoretically mediated microanalysis. J. Chromatogr. A **2004**, *1032*, 173–184.
51. Hadd, A.G.; Raymond, D.E.; Halliwell, J.W.; Jacobson, S.C.; Ramsey, J.M. Microchip device for performing enzyme assays. Anal. Chem. **1997**, *69*, 3407–3412.

52. Hadd, A.G.; Jacobson, S.C.; Ramsey, J.M. Microfluidic Assays of Acetylcholinesterase Inhibitors. Anal. Chem. **1999**, *71*, 5206–5212.
53. Starkey, D.E.; Han, A.; Bao, J.J.; Ahn, C.H.; Wehmeyer, K.R.; Prenger, M.C.; Halsall, H.B.; Heineman, W.R. Fluorogenic assay for b-glucuronidase using microchip-based capillary electrophoresis. J. Chromatogr. B **2001**, *762*, 33–41.
54. Burke, B.J.; Regnier, F.E. Stopped-flow enzyme assays on a chip using a microfabricated mixer. Anal. Chem. **2003**, *75*, 1786–1791.
55. Burke, B.J.; Regnier, F.E. Electrophoretically mediated microanalysis of b-galactosidase on microchips. Electrophoresis **2001**, *22*, 3744–3751.
56. Cohen, C.B.; Chin-Dixon, E.; Jeong, S.; Nikiforov, T.T.A. Microchip-based enzyme assay for protein kinase A. Anal. Biochem. **1999**, *273*, 89–97.
57. Zugel, S.A.; Burke, B.J.; Regnier, F.E.; Lytle, F.E. Electrophoretically mediated microanalysis of leucine aminopeptidase using two-photon excited fluorescence detection on a microchip. Anal. Chem. **2000**, *72*, 5731–5735.
58. Murakami, Y.; Morita, T.; Kanekiyo, T.; Tamiya, E. On-chip capillary electrophoresis for alkaline phosphatase testing. Biosens. Bioelectron. **2001**, *16*, 1009–1014.
59. Lee, M.-Y.; Srinivasan, A.; Ku, B.; Dordick, J.S. Multienzyme catalysis in microfluidic biochips. Biotechnol. Bioeng. **2003**, *83*, 20–28.
60. Tseng, W.-L.; Chang, H.-T.; Hsu, S.-M.; Chen, R.-J.; Lin, S. Immunoaffinity capillary electrophoresis: Determination of binding constant and stoichiometry for antibody-antigen recognition. Electrophoresis **2002**, *23*, 836–846.
61. Chen, S.-H.; Lin, Y.-H.; Wang, L.-Y.; Lin, C.-C.; Lee, G.-B. Flow-through sampling for electrophoresis-based microchips and their applications for protein analysis. Anal. Chem. **2002**, *74*, 5146–5153.
62. Bromberg, A.; Mathies, R.A. Homogeneous immunoassay for detection of TNT and its analogues on a microfabricated capillary electrophoresis chip. Anal. Chem. **2003**, *75*, 1188–1195.
63. Koutny, L.B.; Schmalzing, D.; Taylor, T.A.; Fuchs, M. Microchip electrophoretic immunoassay for serum cortisol. Anal. Chem. **1996**, *68*, 18–22.
64. Chiem, N.; Harrison, D.J. Microchip-based capillary electrophoresis for immunoassays: Analysis of monoclonal antibodies and theophylline. Anal. Chem. **1997**, *69*, 373–378.
65. Ichiki, T.; Ujiie, T.; Shinbashi, S.; Okuda, T.; Horiike, Y. Immunoelectrophoresis of red blood cells performed on microcapillary chips. Electrophoresis **2002**, *23*, 2029–2034.
66. Cheng, S.B.; Skinner, C.D.; Taylor, J.; Attiya, S.; Lee, W.E.; Picelli, G.; Harrison, D.J. Development of a multichannel microfluidic analysis system employing affinity capillary electrophoresis for immunoassay. Anal. Chem. **2001**, *73*, 1472–1479.
67. Wang, J.; Ibáñez, A.; Chatrathi, M.P.; Escarpa, A. Electrochemical enzyme immunoassays on microchip platforms. Anal. Chem. **2001**, *73*, 5323–5327.
68. Lim, T.-K.; Ohta, H.; Matsunaga, T. Microfabricated on-chip-type electrochemical flow immunoassay system for the detection of histamine released in whole blood samples. Anal. Chem. **2003**, *75*, 3316–3321.
69. Wang, J.; Ibáñez, A.; Chatrathi, M.P. Microchip-based amperometric immunoassays using redox tracers. Electrophoresis **2002**, *23*, 3744–3749.
70. Linder, V.; Verpoorte, E.; Thormann, W.; de Rooij, N.F.; Sigrist, H. Surface biopassivation of replicated poly(dimethylsiloxane) microfluidic channels and application to heterogeneous immunoreaction with on-chip fluorescence detection. Anal. Chem. **2001**, *73*, 4181–4189.

71. Linder, V.; Verpoorte, E.; de Rooij, N.F.; Sigrist, H.; Thormann, W. Application of surface biopassivated disposable poly(dimethylsiloxane)/glass chips to a heterogeneous competitive human serum immunoglobulin G immunoassay with incorporated internal standard. Electrophoresis **2002**, *23*, 740–749.
72. Dodge, A.; Fluri, K.; Verpoorte, E.; de Rooij, N.F. Electrokinetically driven microfluidic chips with surface-modified chambers for heterogeneous immunoassays. Anal. Chem. **2001**, *73*, 3400–3409.
73. Rossier, J.S.; Schwarz, A.; Reymond, F.; Ferrigno, R.; Bianchi, F.; Girault, H.H. Microchannel networks for electrophoretic separations, Electrophoresis **1999**, *20*, 727–731.
74. Rossier, J.S.; Girault, H.H. Enzyme linked immunosorbent assay on a microchip with electrochemical detection. Lab On A Chip **2001**, *1*, 153–157.
75. Yakovleva, J.; Davidsson, R.; Lobanova, A.; Bengtsson, M.; Eremin, S.; Laurell, T.; Emnéus, J. Microfluidic enzyme immunoassay using silicon microchip with immobilized antibodies and chemiluminescence detection. Anal. Chem. **2002**, *74*, 2994–3004.
76. Jiang, G.; Attiya, S.; Ocvirk, G.; Lee, W.E.; Harrison, D.J. Red diode laser induced fluorescence detection with a confocal microscope on a microchip for capillary electrophoresis. Biosens. Bioelectron. **2000**, *14*, 861–869.

Received September 1, 2005
Accepted December 14, 2005
Manuscript 6770B

Publication 4

Pharmaceutical applications of isoelectric focusing on microchip with imaged UV detection

Vlčková, M., Kalman, F., Schwarz, M.A.

J. Chromatogr. A 2008, 1181, 145-152

Available online at www.sciencedirect.com
ScienceDirect
Journal of Chromatography A, 1181 (2008) 145–152
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Pharmaceutical applications of isoelectric focusing on microchip with imaged UV detection

Markéta Vlčková [a], Franka Kalman [b], Maria A. Schwarz [a,b,*]

[a] *Department of Chemistry, University of Basel, Spitalstrasse 51, 4056 Basel, Switzerland*
[b] *Solvias AG, WKL-127.2.40, Klybeckstrasse 191, Postfach, 4002 Basel, Switzerland*

Received 4 October 2007; received in revised form 20 November 2007; accepted 18 December 2007
Available online 24 December 2007

Abstract

For the first time, the application of a commercial Shimadzu microchip electrophoresis system MCE-2010 equipped with an imaging UV detector for isoelectric focusing (IEF) of therapeutic proteins is reported. By proper adjustment of the pH gradient, samples with p*I* values ranging from 2.85 to 10.3 can be focused to the imaged part of the separation channel. Three therapeutic proteins (hirudin, erythropoietin, and bevacizumab) have been successfully focused on the microchip, and the results have been compared to conventional capillary IEF in terms of peak profile, p*I* values, and reproducibility.

Keywords: Isoelectric focusing; Microfluidics; Whole-column imaging; Therapeutic proteins; cIEF

1. Introduction

Isoelectric focusing (IEF) is considered to be an attractive separation technique for biologically important amphoteric compounds (e.g. proteins and peptides) due to the high-resolution power and spontaneous focusing of analytes. The analytes are separated on the basis of their isoelectric point (p*I*), either in a pH gradient formed along the separation path by special amphoteric buffers (so-called carrier ampholytes), or, alternatively, in the immobilized pH gradient [1]. During the measurement the sample components are separated and focused at the locations corresponding to their p*I* values. Thereby, the determination of the substance specific p*I* values of the compounds of interest is possible. Thus, this technique is quite often applied to identity tests in pharmaceutical quality control and to the characterization of complex biological samples. Traditionally, IEF is performed in a slab gel, which is a time-consuming and labor-intensive procedure providing only approximate results. However, it remains popular due to the simple technical requirements [1].

Capillary isoelectric focusing (cIEF) offers many advantages over gel-based IEF, for example, ease of automation, quantitation, low consumption of reagents, and fast analysis speed [2–4]. Microchip isoelectric focusing (MC-IEF) is the result of the miniaturization of the cIEF and thus the focusing time and the amount of reagents are further reduced [5]. Since most capillary electrophoretic (CE) instruments and electrophoretic microchip systems are equipped with a single-point, on-column optical detection (UV/vis absorption, fluorescence), all protein bands focused inside the capillary or the microchannel must be mobilized to pass the detection point. Thus, a mobilization step is necessary following the focusing process [6]. Alternatively, the mobilization can commence during focusing if cIEF is performed in uncoated capillaries/channels, but in this case the proteins may be eluted before being focused [7]. Moreover, the adsorption of the proteins to the surface of an uncoated capillary is a severe problem associated with a one-step cIEF method. On the other hand, in the two-step approach, the mobilization step inevitably leads to distortion of the pH gradient resulting in poor reproducibility, longer analysis time and poorer resolution [8].

Whole-column imaging detectors (WCID) proved to be ideal for the detection of focused zones by cIEF as they eliminate the

* Corresponding author at: Department of Chemistry, University of Basel, Spitalstrasse 51, 4056 Basel, Switzerland. Tel.: +41 61 6866523; fax: +41 61 2671005.
E-mail addresses: Maria.Schwarz@solvias.com, Maria.Schwarz@unibas.ch (M.A. Schwarz).

0021-9673/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2007.12.046

need for mobilization. Since the introduction of WCID concept for cIEF, reported by Pawliszyn and coworkers [9], several types of WCID have been developed (for review see [10]). Fluorescent imaging detectors are the most commonly used WCID because of their sensitivity. Besides laser (laser-induced fluorescence, LIF), also organic light emitting diode (OLED) array was used as a light source for WCID [11]. Newly, also a liquid-core waveguide LIF version of WCID, which offers very high detection sensitivity, has been introduced for cIEF [12]. UV absorption imaging detectors are in general less sensitive but can be used for most proteins without a labeling reaction. Recently, a commercial instrument for cIEF equipped with UV–WCID has been launched [13,14]. The WCID concept has also been successfully adapted for microchip format, based on measuring fluorescence [15–17] as well as the UV absorption [5]. In addition to glass chips [5,15], cheaper plastic chips [16,17] have also been applied for MC-IEF–WCID. Furthermore, a microfluidic cartridge suitable for cIEF with WCID has been recently prepared by screen-printing [18] as a novel microfabrication technique. Another benefit of imaged IEF is the possibility of dynamic monitoring the focusing process which provides valuable additional information. Consequently, WCID–IEF has been applied for numerous dynamic measurements, such as for monitoring protein reactions [19,20], for characterization of biomolecular interactions [19,21,22] as well as for measurement of diffusion coefficients [23] or dissociation rate constants of complexes [24].

A commercial Shimadzu microchip electrophoresis system MCE-2010, originally developed for high-throughput DNA analysis, is equipped with a linear imaging UV photodiode array detector. The combination of high throughput and the linear imaging detector makes the instrument attractive for IEF analyses. In spite of this feature, to the best of our knowledge, the instrument has not so far been applied to MC-IEF measurements. The published applications of the MCE-2010 system cover chiral separations [25,26], various analyses (e.g. determination of drugs [27], inorganic metabolites [28], or separation of DNA fragments [29]), and affinity measurements [30,31].

Our goal was to test the suitability of the MCE-2010 system for the fast development of IEF of selected therapeutic proteins, and thus to evaluate the apparatus as a potential high-throughput tool for protein-based formulations. In the pharmaceutical industry, the purity, heterogeneity and identity of the protein-based drugs has to be monitored from development through final lot manufacturing in order to ensure their therapeutic efficacy [32].

Three therapeutic proteins (Revasc Canyon Pharmaceuticals Limited, UK), erythropoietin, and Avastin (Roche Pharma AG, Switzerland)), accepted for treatment of diseases, have been chosen for assessment of the applicability of the MCE-2010 system for MC-IEF measurements. The anticoagulant drug Revasc is a genetically engineered recombinant form of hirudin, which is a natural anticoagulant polypeptide present in leech saliva with a biological effect based on thrombin inhibition [33]. Usually, IEF is used for testing the identity and purity of this therapeutic protein in the quality control. Erythropoietin (EPO) is a glycoprotein hormone that promotes the formation of red blood cells in the bone marrow. This hormone is produced by recombinant DNA technology in mammalian cell culture for treating anemia resulting from chronic renal failure or from cancer chemotherapy. However, the cell line used for EPO production influences its glycosylation, which plays an important role in its biological activity. IEF is a suitable method for measuring the relative proportions of the isoforms present in the EPO sample, as the degree of sialic acid content is reflected by the p*I* value [34,35]. Avastin is trade name of bevacizumab, a humanized monoclonal IgG1 antibody, which is the first commercially available angiogenesis inhibitor. It is used in the treatment of cancer, where it inhibits tumor growth by blocking the formation of new blood vessels by inhibiting the vascular endothelial growth factor [36]. IEF is one of the main methods used in the pharmaceutical industry for the identification of charge variants of monoclonal antibodies such as bevacizumab.

The scope of the present work covers: (i) the evaluation of the applicability of the Shimadzu microchip station MC-2010 for MC-IEF; (ii) the development of the MC-IEF of selected proteins of pharmaceutical interest; (iii) the comparison of the results of MC-IEF with those of conventional cIEF.

2. Experimental

2.1. Chemicals and reagents

The carrier ampholytes BioChemika (pH 3–10) and Pharmalyte (pH 2.5–5) were obtained from Fluka (Buchs, Switzerland). The carrier ampholytes Bio-Lyte, (pH 3–10, pH 6–8, and pH 8–10) were purchased from Bio-Rad Labs. (Reinach, Switzerland). p*I* markers from two sources, from Fluka and from Isogen Life Sciences (IJsselstein, The Netherlands), have been used in the study. Commercial cIEF gel was purchased from Beckman Coulter (Fullerton, CA, USA, ordering no. 477497). All other chemicals used in the experiments were of analytical grade and all aqueous solutions were prepared using deionized water. Solutions of 10 mM and 50 mM phosphoric acid and 20 mM sodium hydroxide, prepared using water as well as gel as a solvent, served as anolytes and catholytes, respectively, for MC-IEF. Commercially available solutions of catholyte (40 mM sodium hydroxide, ordering no. 148-5028), anolyte (20 mM phosphoric acid, ordering no. 148-5029), and cathodic mobilizer (ordering no.148-5030) from Bio-Rad Labs. were used in the cIEF experiments. One percent aqueous solution of hydroxypropylmethylcellulose (HPMC) was used as the medium for cIEF instead of commercial cIEF gel. *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED) was applied to extent the measurable range of the pH gradient on the basic side in some experiments by acting as a spacer.

2.2. Samples

Drugs (Revasc, and Avastin) were bought in the pharmacy. Erythropoietin was provided by the European Pharmacopoeia as biological reference product (BRP). The drug Revasc (batch no. H4007A) is supplied as a pure powder together with a solvent for preparing solution for injection. The powder was diluted by deionized water to final concentration 2 mg/ml and this solution was directly used as a protein sample. Erythropoi-

etin from Pharmacopoeia (batch no. 2b) is a powder containing low-molecular-mass excipients [35]. This powder was dissolved in deionized water to the final EPO concentration of 1 mg/ml and the excipients of low-molecular-mass were eliminated by passage through a Microcon cartridge (Millipore Corporation, Bedford, MA, USA) for 10 min at 13,000 rpm at 4 °C and the retentate was washed three times using deionized water under same conditions. The retentate was then recovered from the cartridge by centrifugation (5 min, 8000 rpm, 4 °C) and directly used as a protein sample. Avastin (batch no. B3048) was supplied as a vial with 4 ml of solution containing 100 mg of bevacizumab and low-molecular-mass excipients. The excipients were again removed by the procedure identical to those used for preparing EPO sample. All proteins samples were stored frozen if they were not analyzed immediately.

The final samples for IEF were prepared by mixing the concentrated solution of ampholytes, p*I* markers, protein sample (if used), and the used medium (cIEF gel or 1% aqueous solution of HPMC, respectively). TEMED was added to the sample as a spacer in some experiments. The concentrations of the sample constituents differ according to a conception of particular experiment and will be given below if necessary.

2.3. Instrumentation and procedures

2.3.1. MC-IEF

On-chip measurements were performed on the commercial Shimadzu microchip electrophoresis system MCE-2010 (Kyoto, Japan). The D2-lamp based instrument possesses a diode array detector with 1024 elements located along the separation channel. The quartz microchip (type D–C), purchased from Shimadzu, has a simple cross design with the channels of 50 μm (depth) × 110 μm (width), and its channels are coated with linear polyacrylamide to suppress the electroosmotic flow. The effective separation length (the imaged separation length) is 25 mm. The schematic picture of the microchip with the respective lengths of the channels is given in Fig. 1. There are four platinum electrodes on the chip to apply voltages between the injection and separation reservoirs located at the end of each channel. These reservoirs are labeled buffer inlet (BI), buffer outlet (BO), sample inlet (SI), and sample outlet (SO).

Prior to the IEF measurement, the BI, SI, and SO reservoirs have been filled with the sample and vacuum has been applied to the BO channel in order to fill the separation channel with the separation mixture. BI reservoir was then emptied and filled with catholyte and BO was filled with anolyte. For MC-IEF, a separation voltage of 1.8 kV (450 V/cm) was applied between the BI and BO. The sample mixture present in the SI and SO reservoirs was drawn back by applying a pinched voltage (varied according to the composition of the sample, from 1.0 to 1.5 kV) to prevent them from entering the separation channel during the IEF. The focusing time depended on the sample composition and will be given separately for each experiment. A wavelength of 280 nm was used for monitoring the progress of the separation, and the final image of the IEF trace was then converted to a data file for further processing. Between measurements, the microchip was rinsed manually with deionized water using a syringe.

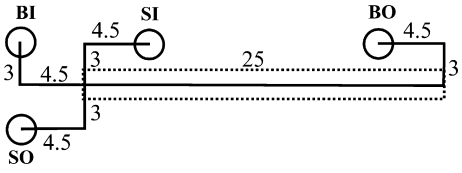


Fig. 1. Design of the microchip used in this study. Dotted rectangle represents the part of the separation channel detected by the diode array. Lengths of the channels are given in mm. Reservoirs: BI, buffer inlet; BO, buffer outlet; SI, sample inlet; SO, sample outlet.

2.3.2. cIEF

A capillary electrophoretic instrument Proteomelab PA 800 from Beckman Coulter (Fullerton, CA, USA) fitted with a filter UV detector set to 280 nm was applied for cIEF measurements. The separations were carried out on commercially available coated capillaries (eCAP Neutral capillary, Beckman) of I.D. 50 μm, with an effective length of 20 cm and total length of 30 cm. The capillary was filled with the sample by applying pressure 25 psi (1 psi = 6894.76 Pa) for 25 s. The separation was run at 25 kV (833 V/m). Under this voltage, first a focusing step took place for 4 min. After replacing the catholyte solution with mobilizer solution, a mobilization step followed with voltage maintained at 25 kV. Between injections, the capillary was rinsed for 1 min with 10 mM phosphoric acid and then for 1 min with deionized water.

3. Results and discussion

3.1. Application of MCE-2010 for MC-IEF measurements

The Shimadzu MCE-2010 microchip station has to be used with the commercial microchips supplied by Shimadzu. All available microchips have a simple cross-injector design and imaged separation length of 25 mm. As the design of the microchip for MCE-2010 (Fig. 1) has been developed for zone electrophoretic measurements, the first task was to assess its applicability for IEF measurements.

The cross-injector is not ideally suited for IEF measurements since the sample present in the sample reservoirs and sample channel can enter the separation channel during the focusing and thereby disturb the separation process. Thus, the undesired flows of the sample during focusing have been prevented by using a pinched voltage applied to the SI and SO reservoirs. The focusing process took place in the whole channel between the BI and BO reservoirs with a total separation length of 40 mm. However, the linear imaging detector of the microchip station covers only a section of the separation channel of 25 mm, starting from the cross-injector onwards, as depicted in Fig. 1. Therefore, the measurable range of the pH gradient, which is focused within the visible part of the separation channel, had to be determined.

Two commercially available broad pH range ampholytes (pH 3–10), BioChemika (high resolution, from Fluka), and Bio-Lyte (from Bio-Rad), have been compared for their IEF performance on the Shimadzu MCE-2010 microchip station (Fig. 2). Surprisingly, the visible part of the pH gradient shifts markedly

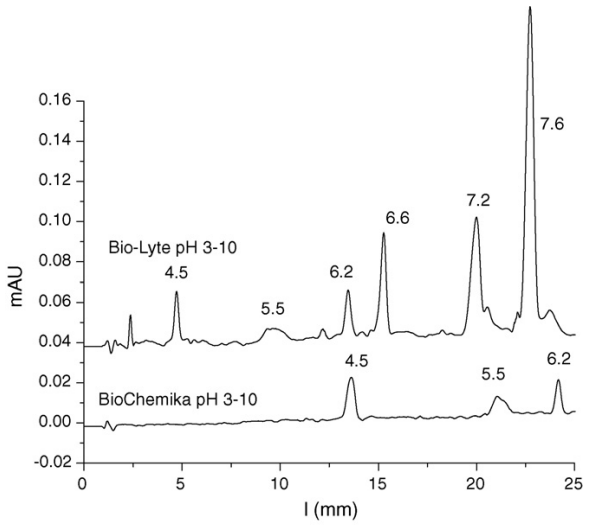


Fig. 2. Comparison of two IEF separations of 8 p*I* markers obtained on MCE-2010 microchip station using different commercially available broad range ampholytes (pH 3–10): BioChemika (high resolution, from Fluka), and Bio-Lyte (from Bio-Rad). Sample solution: 2% (v/v) ampholytes (BioChemika or Bio-Lyte; both 20-fold dilution), 8 p*I* markers (from Fluka, p*I* values: 4.5, 5.5, 6.2, 6.6, 7.2, 7.6, 8.1, 8.7; all 100-fold dilution), medium: cIEF gel. Anolyte: 10 mM H_3PO_4 in water; catholyte: 20 mM NaOH in water; focusing time: 200 s, applied voltages: BI reservoir: 1.8 V, SI and SO reservoirs: 1.2 V, BO reservoir: grounded.

depending on the kind of ampholytes solution used for the measurement with the same mixture of p*I* markers. The markers for this experiment have been selected to cover the pH region, from p*I* 4.5 to p*I* 8.7. However, the p*I* markers with p*I* values higher than 7.6 have not been detected at the end of the focusing process using Bio-Lyte ampholyte and even those with p*I* values higher than 6.2 have not been detected using BioChemika ampholyte. Consequently, BioChemika ampholyte is better suited for measurements of samples with lower p*I* values and, on the other hand, Bio-Lyte ampholyte is advantageous for samples with higher p*I* values. It should be also noted that the visible part of the pH gradient fluctuated to some extent from measurement to measurement, but the pH gradient created by BioChemika was always more shifted to the cathode and thus compounds of lower pH were detected within the imaged part of the channel.

Samples used in the experiment described above have been prepared by diluting the supplied concentrated solution of the respective ampholytes, BioChemika or Bio-Lyte, in the commercial cIEF gel (Beckman), as it was found that better peak shapes of the p*I* markers are obtained in the gel medium compared to water (data not shown). For this reason, a gel medium has been used for preparing all subsequent samples for MC-IEF. On the other hand, water as well as gel, has been used as a medium for preparing the catholyte and anolyte for MC-IEF and the effect of the solvent on the separation process has been studied. The motivation for this investigation was a compression of the pH gradient, which has been described for MC-IEF [11,16,17]. It was observed that on the microchip, the pH gradient is compressed to the middle of the separation channel rather than being uniformly distributed to the whole channel [17]. This compression effect is caused by electrolytes drawing into the microchannels. Higher concentrations of methylcellulose or its derivative have been added to the aqueous sample solution [11] or to the reservoir solutions [17] to increase the viscosity and thus to reduce the compression effect. However, in the present study the compression of the pH gradient during the focusing is advantageous due to the detection arrangement of the Shimadzu MCE-2010 microchip station. As better peak shapes of the p*I* markers have been obtained in a gel (which is more viscous than water) only the medium of the reservoir solutions has been varied. Indeed, the pH gradient was always more compressed when the catholyte and anolyte were prepared in water compared to a gel (Fig. 3a), even though the extent of compression was not perfectly constant. Following from this result, aqueous solutions of catholyte and anolyte have been employed in the majority of following experiments (an exception will be described later) because of their squeezing effect on the pH gradient.

In these experiments, and also later on, p*I* markers from two sources (Fluka and Isogen Life sciences) have been used. The choice between them was made according to their p*I* values to suit a particular analyte. However, mixing of the p*I* markers from Fluka and Isogen in one sample was avoided because the linearity of the pH gradient was worse compared to the linearity achieved if only markers from one source were employed (data not shown).

3.2. Adjustment of the pH gradient

The detection arrangement of the Shimadzu MCE-2010 microchip station means that only the middle part of the separation channel is imaged by the detector. Therefore, a careful adjustment of the pH gradient is required for various applications. Fortunately, a series of narrow range carrier ampholytes is commercially available, which enables the adjustment of the visible part of the pH gradient to a desired range (within some limitations). For example, the use of a mixture of broad range BioChemika with narrow range Pharmalyte (pH 2.5–5) flattens the visible part of pH gradient compared to single broad range BioChemika ampholytes (data not shown), which is advantageous for IEF of proteins having lower p*I* values. The employment of narrow range ampholytes is particularly required for IEF of substances with a markedly low or high p*I* values. However, combining the appropriate narrow range ampholytes with broad range ampholytes is not sufficient for compounds with p*I* values below 3 or above 10. For those, an additional strategy has to be applied to guarantee their focusing within the visible part of the pH gradient.

The addition of TEMED to prevent focusing of basic proteins beyond the detection window in cIEF is a well-known strategy [37,38]. TEMED is a highly basic organic compound and works as a spacer between the catholyte and the basic end of the pH gradient. As a rule of thumb, the ratio of TEMED concentration (%, v/v) to ampholytes concentration should be approximately equal to the ratio of the “non-effective” capillary length to total length [37]. Applying this calculation for the

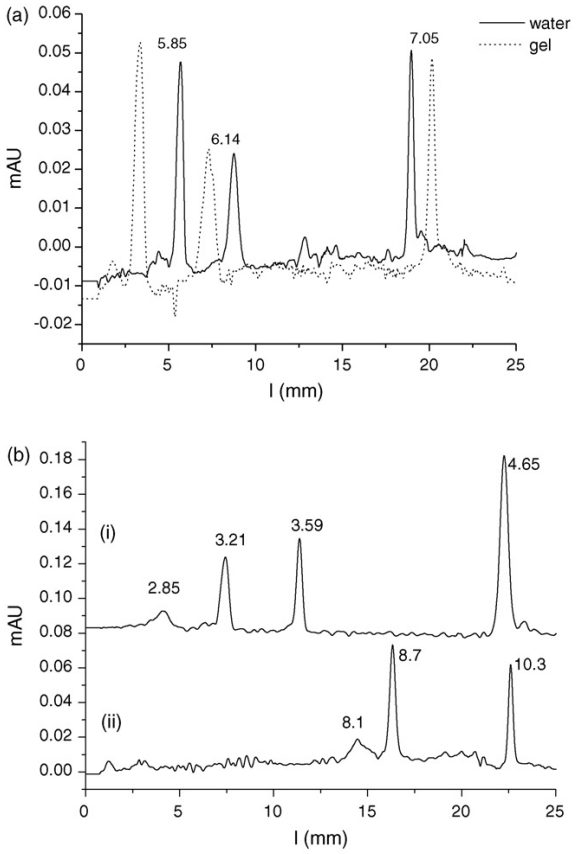


Fig. 3. (a) Comparison of the IEF separations of p*I* markers obtained on MCE-2010 microchip station using anolyte and catholyte dissolved in water (solid line) or in gel (dotted line) for illustration of the compression of pH gradient. Sample solution: 2% (v/v) Bio-Lyte, pH 3–10 (20-fold dilution), 3 p*I* markers (from Isogen; 100-fold dilution), medium: cIEF gel. Anolyte: 10 mM H_3PO_4 in water or gel; catholyte: 20 mM NaOH in water or gel; focusing time: 220 s, applied voltages: BI reservoir: 1.8 V, SI and SO reservoirs: 1.2 V, BO reservoir: grounded. (b) Comparison of the IEF separations of p*I* markers obtained on MCE-2010 microchip station using samples of varied composition in order to show possible adjustment of the pH gradient range detected by the diode array (i) sample solution: 2% (v/v) Pharmalyte, pH 2.5–5 (20-fold dilution), 4 p*I* markers (from Isogen; 100-fold dilution), medium: cIEF gel. Anolyte: 50 mM H_3PO_4 in water; catholyte: 20 mM NaOH in water; focusing time: 300 s, applied voltages: BI reservoir: 1.8 V, SI and SO reservoirs: 1.5 V, BO reservoir: grounded. (ii) Sample solution: 2% (v/v) Bio-Lyte, pH 3–10 (20-fold dilution), 0.4% (v/v) TEMED, 3 p*I* markers (from Fluka; 100-fold dilution), medium: cIEF gel. Anolyte: 10 mM H_3PO_4 in gel; catholyte: 20 mM NaOH in water; focusing time: 300 s, applied voltages: BI reservoir: 1.8 V, SI and SO reservoirs: 1 V, BO reservoir: grounded.

microchip of the Shimadzu MCE-2010 microchip station (total length 40 mm, "non-effective" length 7 mm), the concentration of TEMED which ensures focusing of the proteins into the visible part of the separation channel is 0.375% at 2% ampholytes concentration. Adding 0.4% concentration of TEMED to the sample indeed allowed the detection of the marker with p*I* value of 10.3 (Fig. 3b). However, the necessary prerequisite was to use an aqueous solution of catholyte; using the gel solution of catholyte, the marker focused beyond the imaged area.

On the other hand, there is no analogous substance working as a spacer on the opposite side of the pH gradient. Therefore, another strategy had to be adopted to focus low p*I* substances to the visible part of the separation channel of the Shimadzu microchip. It was observed that the use of a higher concentration of phosphoric acid (which served as anolyte) leads to the shift of the pH gradient in the cathode direction and, thus, enables focusing of compounds with lower p*I* within the visible part of the channel. Using an aqueous solution of 50 mM phosphoric acid and a mixture of BioChemika (pH 3–10) with Pharmalyte (pH 2.5–5) or single Pharmalyte (pH 2.5–5), a marker of p*I* 2.85 has been successfully detected on the microchip (Fig. 3b).

In the summary, applying the described strategies, the measurable p*I* range on the Shimadzu MCE-2010 microchip station can be extended to lower and higher p*I* values than 3 and 10, respectively. This is well comparable to other instruments with imaged optical detection or to standard capillary/microchip electrophoretic instruments.

3.3. Pharmaceutical applications of MCE-2010

To demonstrate the practical applicability of the Shimadzu MCE-2010 microchip station for the pharmaceutical industry, three different therapeutic proteins, hirudin (trade name Revasc), erythropoietin, and bevacizumab (trade name Avastin), respectively, have been investigated by MC-IEF. The focusing of each drug was carried out with three adjacent p*I* markers for reliable determination of their p*I* values by means of linear regression of the respective peak position versus pH. For the optimization of the MC-IEF conditions, the knowledge acquired in the abovementioned experiments has been utilized.

Hirudin is a rather acidic protein with a p*I* value around 4, and so various mixtures of BioChemika (pH 3–10) and Pharmalyte (pH 2.5–5) have been tested for its MC-IEF. The optimized mixture for the separation contained 2% solution of BioChemika and 0.3% solution of Pharmalyte. Under these conditions, hirudin was focused together with three p*I* markers (3.59, 4.65, and 5.85; from Isogen) as a single band into the visible part of the separation channel (Fig. 4a). Its apparent p*I* value has been determined to 3.93 ± 0.02 (Table 1).

EPO is a glycoprotein and consists of several isoforms differing in the degree of glycosylation and in the number of sialic acid residues. Their p*I* values are expected to lie in the acidic region roughly between 3.5 and 4.5. Following from the expected p*I* values of the isoforms, markers of p*I* 2.85, 4.65 and 5.12 (from Isogen) have been chosen to be focused together with EPO in one run. In order to ensure focusing of all selected markers and the protein within the visible part of a separation channel, an aqueous solution of anolyte consisting of 50 mM phosphoric acid has been used, and various mixtures of BioChemika (pH 3–10) and Pharmalyte (pH 2.5–5) have been tested. Using 1% solution of Pharmalyte and 1.6% solution of BioChemika, all of the selected compounds have been successfully focused within the imaged part of the channel (Fig. 4b). Four EPO glycoforms have been unambiguously identified and their apparent p*I* values are summarized in Table 1.

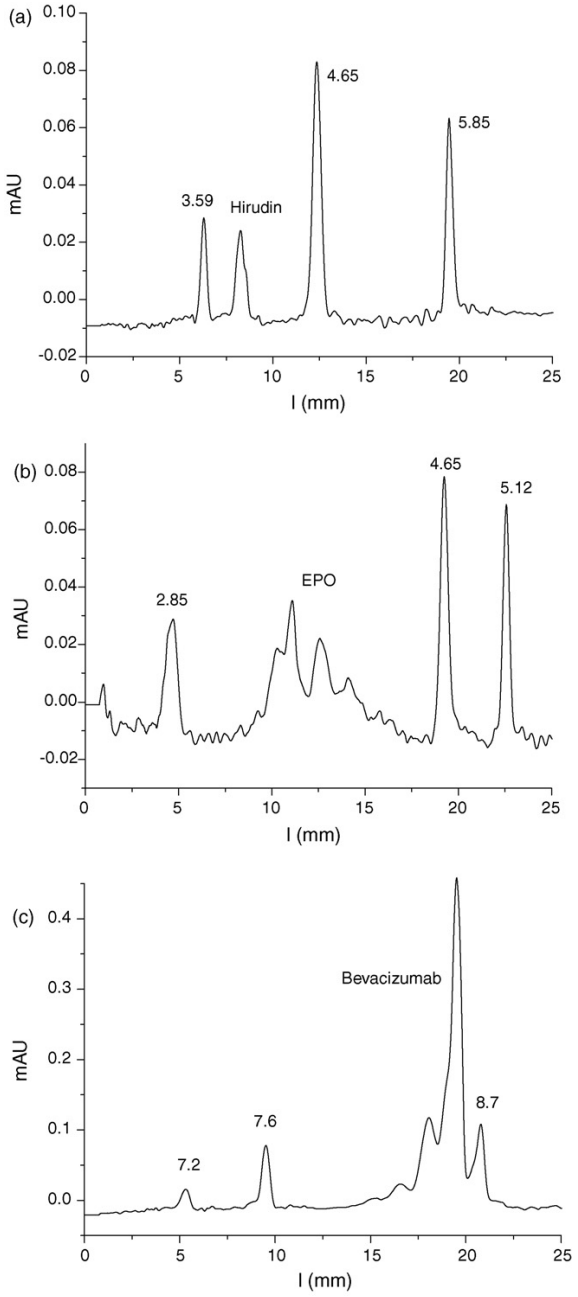


Fig. 4. (a) MC-IEF separation of hirudin (drug Revasc) obtained on MCE-2010 microchip station. Sample solution: 1.6% (v/v) BioChemika, pH 3–10 (25-fold dilution), 1% (v/v) Pharmalyte, pH 2.5–5 (20-fold dilution), 3 p*I* markers (from Isogen), hirudin (0.3 mg/ml), medium: cIEF gel. Anolyte: 10 mM H_3PO_4 in water; catholyte: 20 mM NaOH in water; focusing time: 200 s, applied voltages: BI reservoir: 1.8 V, SI and SO reservoirs: 1.2 V, BO reservoir: grounded. (b) MC-IEF of EPO obtained on MCE-2010 microchip station. Sample solution: 2% (v/v) BioChemika, pH 3–10 (20-fold dilution), 0.3% (v/v) Pharmalyte, pH 2.5–5 (66.67-fold dilution), 3 p*I* markers (from Isogen), EPO (25-fold dilution of retentate), medium: cIEF gel. Anolyte: 50 mM H_3PO_4 in water; catholyte: 20 mM NaOH in water; focusing time: 360 s, applied voltages: BI reservoir: 1.8 V, SI and SO reservoirs: 1.5 V, BO reservoir: grounded. (c) MC-IEF of bevacizumab (drug Avastin) obtained on MCE-2010 microchip station. Sample solution: 2% (v/v) Bio-Lyte, pH 3–10 (20-fold dilution), 0.75% (v/v) Bio-Lyte, pH 6–8 (66.67-fold dilution), 0.375% (v/v) Bio-Lyte, pH 8–10 (66.67-fold dilution), 0.2% (v/v) TEMED, 3 p*I* markers (from Fluka), bevacizumab (50-fold dilution of retentate), medium: cIEF gel. Anolyte: 10 mM H_3PO_4 in gel; catholyte: 20 mM NaOH in water; focusing time: 430 s, applied voltages: BI reservoir: 1.8 V, SI and SO reservoirs: 1 V, BO reservoir: grounded.

Table 1
Comparison of the p*I* determination by MC-IEF and conventional cIEF

Protein	MC-IEF		cIEF	
	Apparent p*I*	RSD (%)[a]	Apparent p*I*	RSD (%)[a]
Hirudin	3.93	0.2	3.91	0.1
Bevacizumab	8.26	0.3	8.27	0.1
	8.45	0.4	8.40	0.2
	8.59	0.2	8.61	0.07
Erythropoietin	3.47	0.5	N/A[b]	N/A[b]
	3.58	0.6		
	3.76	0.3		
	3.94	0.6		

[a] Calculated from four measurements ($n = 4$).
[b] No data obtained using cIEF under conditions analogical to those used in MC-IEF experiments.

Bevacizumab is a humanized monoclonal IgG antibody [36] and p*I* values of its isoforms are expected to be around 8. Therefore, its MC-IEF together with the markers with p*I* 7.2, 7.6 and 8.7 (from Fluka) has been optimized by mixing various portions of the broad range and narrow range ampholytes. The final mixture of the ampholytes consisted of 2% solution of Bio-Lyte (pH 3–10), 0.75% solution of Bio-Lyte (pH 6–8) and 0.375% solution of Bio-Lyte (pH 8–10). The addition of 0.2% TEMED solution to the sample was necessary under these conditions to ensure the focusing of all selected compounds within the visible part of the separation channel. For this application, a gel solution of anolyte was used in order to suppress the effect of pH gradient compression from the acidic side. The catholyte solution was aqueous as usual. Under these conditions three isoforms of bevacizumab have been detected, one main basic isoform and two minor acidic isoforms (Fig. 4c). The apparent p*I* values of the respective isoforms are given in Table 1.

The sensitivity of the linear imaging UV detector of the MCE-2010 station for MC-IEF of selected therapeutic proteins has not been investigated in this study. However, to get the idea of the detector sensitivity, the limit of detection for hirudin has been estimated to 0.01 mg/ml from a signal to noise ratio 3/1. The relatively low sensitivity of the absorption detection is a general drawback of the microchip-based separations due to the small channel depths typically used on the chip [39]. Another potential drawback of the detection arrangement of the MCE-2010 is the unfavorable output of the detection signal, as only the final image of the detector (i.e. image shot at the time specified beforehand) can be converted to a data file for further processing. The progress of the separation is monitored in the real-time on the screen and may be stored as bit map files only. Thus, several runs have been needed to find the optimal separation time.

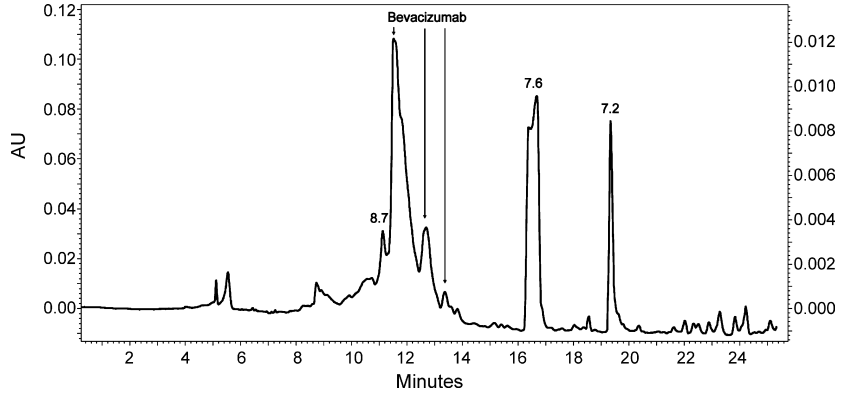


Fig. 5. cIEF separation of bevacizumab (drug Avastin) for comparison with MC-IEF (see Fig. 4c). Sample solution: composition the same as in Fig. 4c, except for medium: here 1% aqueous solution of HPMC. Other conditions are given in Section 2.

3.4. Comparison of MC-IEF with conventional cIEF and with the literature

To confirm the reliability of the IEF measurements on MCE-2010, comparative experiments with the same protein samples have been carried out using conventional cIEF. Each protein has been focused by cIEF under conditions analogical to those optimized for the MC-IEF separation. Only the gel medium has been substituted by 1% aqueous solution of HPMC. This does not interfere with the mobilization step, which necessarily follows the focusing step in cIEF.

The results of cIEF have been compared with the results achieved by MC-IEF regarding the peak profile, apparent p*I* values, and reproducibility (Table 1). Well comparable peak profiles and apparent p*I* values have been obtained for hirudin as well as for bevacizumab using both techniques. As an example, the cIEF of bevacizumab is given in Fig. 5. In contrast, EPO under non-denaturing conditions (without addition of urea) was successfully focused only on the microchip. The separation of the same sample failed in the capillary. The precipitation of the protein under the conditions of cIEF (focusing followed by chemical mobilization) is the most probable cause of this failure as EPO is rather hydrophobic. On the contrary, MC-IEF with imaged detection does not require the mobilization step and enables also a direct control of the optimal focusing time (time that allows for completion of the focusing before precipitation takes place), owing to the real-time detection. That is most probably the reason why MC-IEF of EPO was successful even without the addition of solubilizer.

The four EPO isoforms separated using MC-IEF are in a good agreement with the published results for this protein [40], even though some minor glycoforms have been detected in addition to the four main forms using cIEF [34,35]. However, in these cIEF measurements, urea was added to the sample in order to improve the resolution and without urea, the separation of isoforms failed [34]. This is similar to our results obtained by cIEF. Urea is widely used in IEF to enhance the solubility of hydrophobic proteins but, on the other hand, it denatures proteins, which causes shifts in their apparent p*I* values [8]. For EPO analysis, the effectivity of urea addition was attributed to deaggregation and disruption of hydrophobic and non-covalent interactions [40]. The IEF of EPO without addition of urea is more appropriate in pharmaceutical industry, as possible conformation changes of the protein may be reflected better in such an analysis.

Recombinant hirudin has been previously focused only using slab gel IEF. The p*I* value of this protein reported by Bischoff et al. is 4.3 [41] and, more recently reported by Bossi et al. is 4.03 [33]. In both publications, the p*I* value was determined using IEF in immobilized pH gradients. Moreover, Bossi et al. detected also four other minor components originating from degradation of hirudin. However, in that measurement, a novel recombinant hirudin variant (HM2) produced in *Escherichia coli* was investigated [33]. Hirudin from a pharmaceutical preparation, used in our measurements, was expressed in yeast (*Saccharomyces cerevisiae*, strain TR 1456) and was homogeneous. IEF of bevacizumab was not published yet to the best of our knowledge. However, the peak profile of this humanized monoclonal IgG corresponds well to the typical peak profiles of other therapeutic recombinant IgGs from the literature [14,42].

The reproducibility of p*I* determination using MC-IEF and cIEF has been assessed by two independent sample preparations, each measured twice. As expected, the RSD of cIEF are generally lower than those achieved by microchip IEF. This is a general drawback of the microchip-based measurements versus capillary but it is compensated by the higher throughput of the chip [31].

4. Conclusions

For the first time, a commercial Shimadzu microchip electrophoresis system MCE-2010 has been applied to MC-IEF of proteins of pharmaceutical interest. Although MCE-2010 was originally developed for zone electrophoresis measurements, its features unfavorable for IEF (i.e. cross-injection, and only a part of the separation channel imaged) can be well compensated. A pinched voltage applied to the sample reservoirs prevents the sample from entering the separation channel during IEF. Proper adjustment of the pH gradient ensures focusing of the zones

with broad range of p*I* values (2.85–10.3) to the imaged part of separation channel.

On the one hand, the versatile utilization of the MCE-2010 microchip station could be regarded as a benefit compared to other instruments with imaged detection developed for high-throughput IEF measurements (e.g. commercially available iCE280 Analyzer [13,14]), which are mono functional. On the other hand, the necessary adjustment of the pH gradient for various applications on the microchip station makes the optimization of IEF method more complicated and time consuming compared to the specialized instruments. To sum up, MCE-2010 apparatus may be a good choice for laboratories that need to perform variety of electrophoretic measurements (including some IEF experiments). Certainly, a specialized apparatus, i.e. iCE280 Analyzer, is preferable for laboratories focused on IEF measurements, because iCE280 Analyzer was directly developed to meet all the demands of high-throughput IEF.

Three therapeutic proteins (hirudin, EPO, and bevacizumab) from pharmaceutical formulations have been successfully focused on the MCE-2010 microchip and the results have been compared to conventional capillary IEF. p*I* values as well as the peak profiles, which compare well using both techniques, have been obtained for hirudin and bevacizumab. Erythropoietin under non-denaturing conditions (without addition of urea) was successfully focused only on the microchip. Its peak profile, as well as the calculated apparent p*I* values are in a good agreement with published data [34]. The reproducibility of the determination of p*I* values is somewhat lower on the microchip but the analysis is much faster compared to conventional cIEF, which requires mobilization step after finishing focusing. Moreover, a precise tuning of the focusing time is possible on the microchip as the progress of the focusing could be monitored on the real-time basis.

Acknowledgements

Partial funding for this project provided by Swiss National Science Foundation, grant number 200020-112190, is gratefully acknowledged. The authors also thank the Analytical Department of Solvias AG for providing time and measurement equipment.

References

[1] R. Westermeier, Electrophoresis in Practice, Wiley-VCH Verlag, Weinheim, 2001, p. 45.
[2] K. Shimura, Electrophoresis 23 (2002) 3847.
[3] F. Kilar, Electrophoresis 24 (2003) 3908.
[4] P.G. Righetti, C. Gelfi, M. Conti, J. Chromatogr. B 699 (1997) 91.
[5] Q.L. Mao, J. Pawliszyn, Analyst 124 (1999) 637.
[6] T.J. Pritchett, Electrophoresis 17 (1996) 1195.
[7] X. Liu, Z. Sosic, I.S. Krull, J. Chromatogr. A 735 (1996) 165.
[8] J.Q. Wu, S.C. Li, A. Watson, J. Chromatogr. A 817 (1998) 163.
[9] J.Q. Wu, J. Pawliszyn, Anal. Chem. 64 (1992) 2934.
[10] X.Z. Wu, T.M. Huang, Z. Liu, J. Pawliszyn, TrAC, Trends Anal. Chem. 24 (2005) 369.
[11] B. Yao, H.H. Yang, Q.L. Liang, G. Luo, L.D. Wang, K.N. Ren, Y.D. Gao, Y.M. Wang, Y. Qiu, Anal. Chem. 78 (2006) 5845.
[12] Z. Liu, J. Pawliszyn, Anal. Chem. 75 (2003) 4887.
[13] X.Z. Wu, J.Q. Wu, J. Pawliszyn, LC–GC 19 (2001) 526.
[14] N. Li, K. Kessler, L. Bass, D. Zeng, J. Pharm. Biomed. Anal. 43 (2007) 963.
[15] C. Das, Z. Xia, A. Stoyanov, Z.H. Fan, Instrum. Sci. Technol. 33 (2005) 379.
[16] C. Das, Z.H. Fan, Electrophoresis 27 (2006) 3619.
[17] H.C. Cui, K. Horiuchi, P. Dutta, C.F. Ivory, Anal. Chem. 77 (2005) 1303.
[18] T.M. Huang, P. Ertl, X.Z. Wu, S. Mikkelsen, J. Pawliszyn, Sens. Mater. 14 (2002) 141.
[19] T. Bo, J. Pawliszyn, J. Sep. Sci. 29 (2006) 1018.
[20] Z. Liu, J. Pawliszyn, J. Proteome Res. 3 (2004) 567.
[21] T. Bo, J. Pawliszyn, J. Chromatogr. A 1105 (2006) 25.
[22] T. Bo, J. Pawliszyn, Electrophoresis 27 (2006) 852.
[23] Z. Liu, T. Lemma, J. Pawliszyn, J. Proteome Res. 5 (2006) 1246.
[24] Z. Liu, A.P. Drabovich, S.N. Krylov, J. Pawliszyn, Anal. Chem. 79 (2007) 1097.
[25] F. Kitagawa, S. Aizawa, K. Otsuka, Anal. Sci. 21 (2005) 61.
[26] M. Ludwig, F. Kohler, D. Belder, Electrophoresis 24 (2003) 3233.
[27] E. Guihen, G.D. Sisk, N.M. Scully, J.D. Glennon, Electrophoresis 27 (2006) 2338.
[28] T. Miyado, Y. Tanaka, H. Nagai, S. Takeda, K. Saito, K. Fukushi, Y. Yoshida, S. Wakida, E. Niki, J. Chromatogr. A 1109 (2006) 174.
[29] Z.Q. Xu, Y. Nakamura, T. Hirokawa, Electrophoresis 26 (2005) 383.
[30] T. Le Saux, H. Hisamoto, S. Terabe, J. Chromatogr. A 1104 (2006) 352.
[31] A.R. Stettler, M.A. Schwarz, J. Chromatogr. A 1063 (2005) 217.
[32] K. Ahrer, A. Jungbauer, J. Chromatogr. B 841 (2006) 110.
[33] A. Bossi, P.G. Righetti, C. Visco, U. Breme, M. Mauriello, B. Valsasina, G. Orsini, E. Wenisch, Electrophoresis 17 (1996) 932.
[34] A. Cifuentes, M.V. Moreno-Arribas, M. de Frutos, J.C. Diez-Masa, J. Chromatogr. A 830 (1999) 453.
[35] P. Lopez-Soto-Yarritu, J.C. Diez-Masa, A. Cifuentes, M. de Frutos, J. Chromatogr. A 968 (2002) 221.
[36] W.C. Weinberg, M.R. Frazier-Jessen, W.J. Wu, A. Weir, M. Hartsough, P. Keegan, C. Fuchs, Cancer Metastasis Rev. 24 (2005) 569.
[37] D. Mohan, C.S. Lee, J. Chromatogr. A 979 (2002) 271.
[38] T. Manabe, H. Miyamoto, K. Inoue, M. Nakatsu, M. Arai, Electrophoresis 20 (1999) 3677.
[39] S. Gotz, U. Karst, Anal. Bioanal. Chem. 387 (2007) 183.
[40] A. Pantazaki, M. Taverna, C. Vidal-Madjar, Anal. Chim. Acta 383 (1999) 137.
[41] R. Bischoff, D. Roecklin, C. Roitsch, Electrophoresis 13 (1992) 214.
[42] S. Tang, D.P. Nesta, L.R. Maneri, K.R. Anumula, J. Pharm. Biomed. Anal. 19 (1999) 569.

Publication 5

Microchip isoelectric focusing applications

Vlčková, M., Schwarz, M.A.

Chimia 2008, 62, 244-248

doi:10.2533/chimia.2008.244 CHIMIA **2008**, *62*, No. 4

Chimia 62 (2008) 244–248

ISSN 0009–4293

Microchip Isoelectric Focusing Applications

Markéta Vlčková[§a] and Maria A. Schwarz[*a,b]

[§]SCS Mettler-Toledo Award Winner (Oral Presentation)

Abstract: This review summarizes all available publications on isoelectric focusing carried out on planar microfabricated devices. Characteristic features of microchip isoelectric focusing (MC-IEF), such as resolution independence of separation length and compression of pH gradient, are discussed. An overview of materials used for microchip fabrication and developed detection strategies is given. Accomplishments in on-chip coupling of MC-IEF with other electrophoretic separation techniques or with mass spectrometry are briefly described. The review ends with alternative approaches to MC-IEF separation in terms of unusual sample introduction, kinds of support used for IEF separation, and in manner in which the pH gradient is generated.

Keywords: Electrophoresis · Isoelectric focusing · Microfluidics · Proteomics · Whole-column imaging detection

1. Introduction

Isoelectric focusing (IEF) is an electrophoretic separation technique with high resolution power used to separate amphoteric compounds, in particular peptides and proteins.[1] The amphoteric compounds are separated on the basis of their isoelectric point (pI) in a pH gradient formed by a mixture of special amphoteric buffers, so-called ampholytes. Various wide-pH-range and narrow-pH-range ampholytes mixtures from different suppliers are commercially available in order to optimize a given IEF separation.

The ampholytes build the pH gradient in the electric field, which is applied along a slab gel, a capillary or, more recently, along a separation channel on the microchip. Before the electric field is applied, the separation device is filled with a solution of uniform pH which contains a mixture of ampholytes and a sample. The electrode reservoirs are filled with an acid and a base, respectively. Upon application of the electric field, the negatively charged ampholytes move towards the anode, and the positively charged ones to the cathode. They gradually align themselves between the cathode and the anode according to their pI values, and form a stable pH gradient. Simultaneously, sample components (proteins, peptides) are separated and focused to those positions of the pH gradient which correspond to their pI values. Thus, an estimation of the pI of the unknown sample components is possible with the help of pI markers of known pI values (available commercially). The experimental set-up of IEF performed in the narrow channel format (capillary, microchip) is schematically depicted in Fig. 1a.

Capillary isoelectric focusing (cIEF) offers many advantages over gel-based IEF, such as ease of automation, quantitation, lower consumption of reagents and faster analysis speed.[2] Microchip isoelectric focusing (MC-IEF) results from the miniaturization of the cIEF and so the amount of reagents is further drastically reduced.[3] Moreover, MC-IEF may be on-chip coupled with other processes, which can substantially simplify and speed up the analysis of complex samples.[4] However, most of the commercial instruments for cIEF, and also certain MC-IEF configurations, rely on single-point, on-column optical detection (UV/VIS absorption, fluorescence) and hence do not enable direct detection of the focused proteins (unlike gel-based IEF). For the detection of the focused zones, the focusing process must be followed by a mobilization step. During mobilization all protein bands focused inside the capillary or the micro-channel are moved past the detection point. However, the mobilization leads to distortion of the pH gradient resulting in poor reproducibility, longer analysis time and poorer resolution.[5] To overcome this obstacle, various whole-column imaging detectors for cIEF and MC-IEF have been developed.[2,6] A charged-coupled camera of such a detector images the entire capillary or separation channel, often in real time, and hence the need for mobilization is eliminated. Another benefit of imaged IEF is the possible monitoring of the focusing process, which provides valuable additional information.[2]

Since the first demonstration of IEF on the microchip in 1999,[3,7] a lot of effort has been put into the development of MC-IEF for various applications. This review gives an overview of all applications of MC-IEF which have been hitherto published. The classification of the applications into the sections in this review is based on new ac-

*Correspondence: Dr. M. A. Schwarz[a,b]
Tel.: + 41 61 686 6523
Fax: + 41 61 267 1005
E-mail: Maria.Schwarz@unibas.ch,
Maria.Schwarz@solvias.com
[a]Department of Chemistry
University of Basel
Spitalstrasse 51
CH-4056 Basel
[b]Solvias AG
WKL-127.2.40
Klybeckstrasse 191
Postfach
CH-4002 Basel

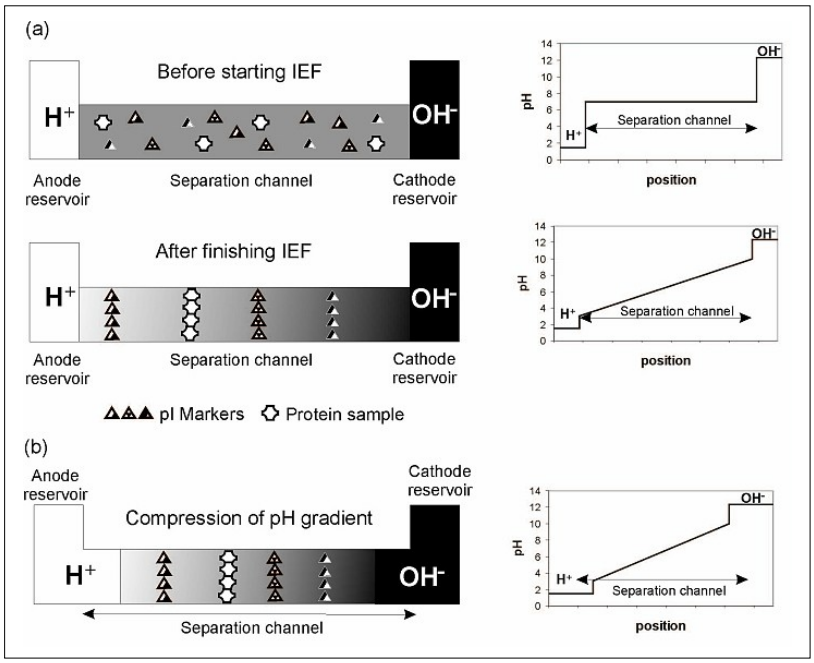


Fig. 1. Experimental set-up of IEF. Schematic drawing of (a) IEF process performed in a narrow channel format; (b) compression of pH gradient occurring during MC-IEF.

complishments, rather than on the type of analyzed protein. The first section covers first realizations of MC-IEF and contains three sub-sections referring to MC-IEF phenomena, detection systems for MC-IEF, and available chip materials, respectively. A section is devoted to applications dealing with on-chip integration of MC-IEF with other processes. Finally, the alternative approaches to MC-IEF are summarized in a separate section. The review ends with conclusions and a short outlook to the future.

2. Realization of MC-IEF

The early applications of MC-IEF were mostly aimed at the demonstration of feasibility of IEF separation on a microfluidic device. Hofmann *et al.* constructed a glass microchip for MC-IEF and compared three common mobilization methods.[7] The electroosmotically driven mobilization occurring simultaneously with the focusing has been suggested for using with microchip due to its speed, compatibility with electroosmotic flow (EOF), and minimum instrumentation requirements. Concurrently, MC-IEF on the chip with UV absorption imaging detection has been demonstrated by Mao and Pawliszyn.[3] The channels of the quartz chip applied for the IEF separation had to be coated with linear polyacrylamide to achieve good reproducibility and resolution. More recently, MC-IEF was performed on a plastic device;[8] single-point LIF detection together with chemical mobilization was used for this MC-IEF separation. The same device has been also successfully applied to the study of protein–protein interactions causing a pI value change.[8] Fast MC-IEF separation carried out on a plastic chip with ultra-short separation channel has been demonstrated by Han and Singh.[9] Using a microchannel 4 mm long, IEF could be completed within 45 s without sacrificing the resolution. The resolution was not lowered while shortening the channel (at constant voltage), as follows from the resolution phenomenon occurring in IEF (see below).

2.1. *MC-IEF Phenomena*

During the first miniaturization efforts, it was confirmed experimentally as well as theoretically, that in IEF separations, the resolution does not depend on the separation length.[8–10] Das and Fan studied this phenomenon in MC-IEF in detail.[10]

The minimum difference in pI values required for two proteins to be separated ($\Delta(pI)_{min}$) can be given by the following Eqn. first reported by Vesterberg and Svensson:[11]

$$\Delta(pI)_{min} = 3\sqrt{\frac{D \cdot (d(pH)/dx)}{E \cdot (-d\mu/d(pH))}} \qquad (1)$$

where D is the diffusion constant of the protein, d(pH)/dx is the pH gradient, E is electric field strength, and dμ/d(pH) is the mobility slope at each pI value. The following assumptions have been used in the derivation of Eqn. (1). First, pH gradient is continuous and the applied electric field is constant. Second, both proteins have the same diffusion coefficient. Third, the two adjacent proteins are considered separated when the positions of their peak maximums differ by three standard deviations. Fourth, there are no effects resulting from EOF, hydrodynamic siphoning, and other such movements. Finally, there is no Joule heating due to high electric currents.

When a constant voltage, V, is applied and a uniform pH gradient is used, then E = V/L and d(pH)/dx = ΔpH/L, where L is separation length. Consequently, Eqn. (1) may be simplified to Eqn. (2):

$$\Delta(pI)_{mjn} = 3\sqrt{\frac{D \cdot \Delta pH}{V \cdot (-d\mu/d(pH))}} \qquad (2)$$

in which there is no term related to the distance. By shortening the channel length, a higher electric field is applied when the voltage remains the same and the peaks become sharper. Consequently, the IEF separation resolution is not sacrificed (at the same voltage V) with the decreasing channel length. Naturally, the application of a higher separation voltage leads to a higher resolution (lower $\Delta(pI)_{min}$, see Eqn. (2)). Additionally, a linear relationship between the focusing time and the inverse of the electric field strength has been established.[10]

Regarding the resolution dependence on separation voltage, Stoyanov *et al.*[12] have demonstrated that above a certain electric field (similar to 300 V/cm), the resolution does not increase with the increasing voltage anymore and even lowers. This observation has been ascribed to the detrimental effects of Joule heating at higher electric field, which has led to a band-broadening and breakdown of a gel used to minimize the EOF.

Another phenomenon relating to MC-IEF is the compression of pH gradient, which was for the first time reported by Cui *et al.*[13] With the help of imaged detection it was observed that the pH gradient in a microfluidic device is compressed to the middle of a channel rather then uniformly distributed in the whole channel (Fig. 1b). This compression effect is caused by electrolytes drawing into the microchannels and the degree of compression decreases with the channel length.[10] High concentrations of methylcellulose or its derivative have been added to the aqueous sample solution[6] or to the reservoir solutions[13] to increase the viscosity and thus to reduce the compression effect.

2.2. *Detection for MC-IEF*

The following three detector types for MC-IEF may be distinguished: single-point on-column detectors, whole-column imaged detectors, and special off-column detectors, respectively. Obviously, most efforts have been devoted to the development of whole-column imaged detection, as the problematic mobilization of the focused zones could be circumvented with this detector type. Moreover, the planar arrangement and short separation length make the practical realization of the imaged detection less demanding compared to cIEF. Imaged UV absorption detectors[3,14] as well as various imaged fluorescence detectors have been developed for MC-IEF. In addition to lamp[15] and laser (laser-induced fluorescence detection),[16,17] also organic light emitting diodes[6] have been used as the light source for fluorescence detection. The imaging is usually realized by focusing the light on the whole channel and sensing through charged-coupled devices (CCD). Alternatively, a fast scanning fluorescence detector, moving along the channel, has been applied for MC-IEF.[15,16]

The single-point on-column detectors measure mostly fluorescence.[7,8] In addition to fluorescence, a selective and sensitive chemiluminiscence detector has been developed for single-point detection of heme proteins separated by MC-IEF.[18] In relation to the single-point detection, a novel pressure on-chip mobilization using a diaphragm pump has been reported,[19] which enables a precise control of the mobilization and facilitates the connection of the IEF to second dimension separation technique.

Special off-column detection represents the demanding connection of MC-IEF to a mass spectrometer (MS). MC-IEF was successfully on-chip coupled to electrospray ionization (ESI), followed by ion-trap MS, as demonstrated by Wen *et al.*[20] Recently, MC-IEF has been coupled with matrix-assisted laser desorption/ionization (MALDI) time-of-flight MS, using a pseudo-closed channel as an interface.[21] The pseudo-closed channel, which served for MC-IEF separation, was formed between two plastic wafers, the base and the cover, respectively. After removal of the cover, a buffer solution was evaporated from the base, and then the matrix solution was added. In this way the laser reached the separated proteins directly on-chip without an elution step. In a similar approach demonstrated by Fujita *et al.*,[22] a chip covered with removable resin tape has been applied for MC-IEF. After finishing the MC-IEF separation, the chip was immediately frozen in order to fix separated proteins in their position. Then the tape was removed, matrix solution was added, and the detection by MALDI-MS was performed.

The connection of MC-IEF with MS might be considered as a special case of on-chip integration of more processes as it enables a high-throughput two dimensional (2D) mapping. In the first dimension, sample proteins are analyzed in terms of their pI by MC-IEF, and in the second dimension, the samples are measured in terms of their mass-to-charge ratio by MS.[22]

2.3. *Chip Materials*

Besides traditional glass chips, MC-IEF separations have also been often performed in cheaper plastic chips and, alternatively also in combined glass/plastic chips (one layer glass, second layer plastic[6,18,19]). In the case of plastic chips, poly(dimethylsiloxane) (PDMS) is the most often used polymeric material,[9,13,23] but the use of other polymeric materials such as poly(methyl metacrylate) (PMMA)[8,24] or polycarbonate (PC)[23,25] has also been reported.

An interesting alternative material for chip fabrication for MC-IEF is cyclic olefin copolymer (COC) due to its ease of fabrication, low cost and solvent resistance.[26] The unfavorable protein adsorption normally associated with this material was suppressed by covering the surface of COC device by polyacrylamide.[26] Covering the inner chip walls by polyacrylamide increased the hydrophilicity of the walls, and at the same time suppressed also the EOF, as desired for MC-IEF measurements with imaged detection. Alternatively, the EOF on COC device was kept low by dynamic coating using a mixture of hydroxyethylcellulose and hydroxypropylcellulose.[10] To further suppress protein adsorption onto this device, only the second run of MC-IEF separation was evaluated.

The glass chips for the MC-IEF are usually coated, either dynamically[18] or permanently using polyacrylamide.[3,27] An unusual approach to the permanent coating is the modification of the glass surface using plasma-polymerized films, as reported by Tsai *et al.*[28] Acetonitrile as well as hexamethyldisiloxane (HMDS) monomers were used for plasma polymerization to modify the glass surface; the stability of the coating was substantially better in case of hydrophobic HMDS films.[28]

3. On-chip Integration of MC-IEF with other Processes

One of the attractive features of chip technology is the potential of integration of more processes into one device with the goal to eventually develop a so-called micro-total analysis system (μ-TAS), a miniaturized flow system for fast, fully automated analysis. Due to the relative newness of MC-IEF technology, MC-IEF has so far been coupled only to another electrophoretic separation technique or to the MS detector. As the connection of MC-IEF with MS has already been described in one of the previous sections (detection for MC-IEF), this section summarizes available reports on coupling of MC-IEF to another electrophoretic process.

The integration of MC-IEF with other electrophoretic technique was mostly aimed at development of a 2D separation device with two independent separation mechanisms, which are employed sequentially. Typically, the MC-IEF step serves as the first dimension followed by another electrophoretic separation step, which can either be free solution electrophoresis[29,30] or gel electrophoresis.[24,25,30] The most challenging task is the transfer of proteins between the two dimensions, which is achieved through a special design of the chip in combination with a complicated voltage control[24,25,29] or, alternatively, with a pressure control using a system of microvalves.[30]

A slightly different approach to the on-chip integration of more processes represents the coupling of several stages of MC-IEF separation in series.[31] First, proteins are focused in a straight channel using broad-range ampholytes. Then, segments of the first channel are refocused in secondary channels branched from the first channel at T-junctions. A significantly higher resolution was achieved during refocusing of the tested proteins in the secondary channels due to shallower pH gradient and higher electrical field gradient in comparison to the MC-IEF separation in the first channel.

4. Alternative Approaches to MC-IEF

In addition to the standard set-up of MC-IEF (summarized in Fig. 1), a number of MC-IEF separations with non-standard arrangement of the IEF process have been developed. The unusual approaches to MC-IEF differ from the classical arrangement:

i) in terms of sample introduction;
ii) in the kind of separation device used for IEF separation;
iii) in the manner of generation of pH gradient.

i) In a standard MC-IEF arrangement, the channel is filled with the mixture of ampholytes and sample before starting the focusing process. An alternative, dynamic sample introduction for MC-IEF has been developed in order to increase sample loading and thereby also the concentration of the analytes in the focused zones.[23] In this dynamic approach, the analytes (proteins or peptides) are initially present in electrode reservoirs and during the separation continuously migrate into the channel, encounter the pH gradient established

by the ampholytes and undergo the focusing. Sample loading capacity can be directly controlled by the injection time and the applied electric field strength; however the discrimination between the analytes by electrokinetic injection cannot be avoided (the loading of analyte with higher electrophoretic mobility will be greater compared to the analyte with lower mobility).

ii) The standard MC-IEF separation takes place in a narrow separation microchannel. In contrast, a flat bed is used for separation in the method of *free flow MC-IEF*, introduced by Xu *et al.*[32] In free flow IEF, a wide stream of a mixture of ampholytes and sample is continuously admitted at one end of the separation bed under laminar pressure flow conditions. An electric field is applied perpendicular to the pressure driven flow with the help of side channels. These side channels connect the separation bed with electrode compartments filled with an acid and a base, respectively, and serve for applying an electric field (Fig. 2a). Sample components migrate through the separation bed transversally until they reach their isoelectric points and thereafter form narrow parallel lines. Consequently, free flow MC-IEF is applied mainly to increase the concentration of the analytes. However, the side channels cause a significant potential loss and a loss of part of the pH gradient due to the spreading of ampholytes to them. To overcome this drawback, an improved device for free-flow MC-IEF has been developed by Kohlheyer *at el.*[33] Two photopolymerizable membranes fabricated directly in the chip isolate the separation bed from the electrodes, yet allow the electrical contact due to their ion permeability. The design of the device has been further improved by employing multiple sheath flows and preseparated ampholytes, leading to a higher resolution and peak capacity of the free flow MC-IEF separation.[34]
Another non-standard separation device is utilized in so-called *parallel IEF* developed by Zilberstein *et al.*[35–37] In the specially designed parallel device, the pH gradient is not created along the electric field, but across a membrane with a large amount of microscopic channels filled by gel droplets with different pH values (Fig. 2b). During the separation, analytes are trapped in the droplets with pH corresponding to their pI values, which is a favorable feature for preparative separations.

iii) The pH gradient for the IEF process is normally created in the electric field by the mixture of ampholytes. In an alternative approach, water electrolysis has been utilized for the formation of the pH gradient in a microfluidic device developed by Macounova *et al.*[38,39] The separation channel of this device has the two opposite walls formed by gold or palladium electrodes. Upon application of an electric field to the electrodes, water electrolysis occurs and an *electrically generated pH gradient* is formed across the channel. The proteins are admitted to the channel in pressure-driven flow and are separated by means of transverse IEF (Fig. 3a). Another non-standard approach represents a *thermally generated pH gradient*, which is formed in the electric field along a tapered separation channel filled with a buffer[27] (Fig. 3b). Nonuniform Joule heating is generated along the tapered channel, which causes the formation of a *pH gradient* by the buffer as its dissociation constant depends on the temperature.

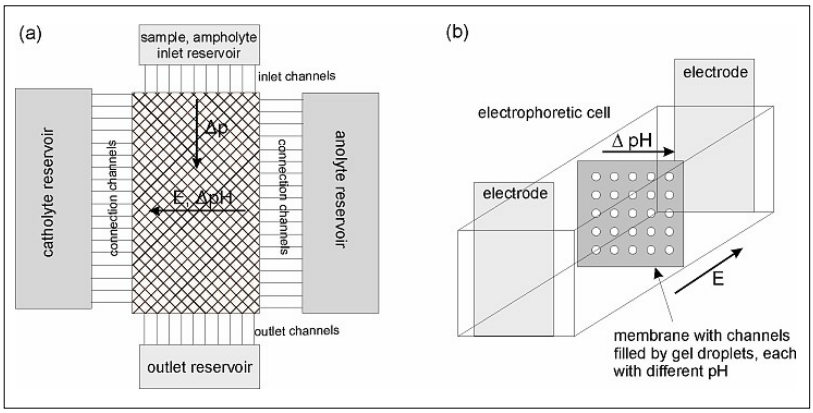


Fig. 2. Non-standard approaches to MC-IEF. Schematic drawing of (a) free flow MC-IEF; (b) parallel MC-IEF; the arrows indicate the direction of pressure gradient (Δp), electric field (E), and pH gradient (ΔpH).

5. Conclusions and Outlook

The feasibility of performing IEF on microchip has been demonstrated and MC-IEF has become a new emerging analytical technology with a rapidly growing number of applications. Compared to the miniaturization of other electrophoretic techniques, the adaptation of IEF to microchip has the advantage of not being hindered by compromised resolution due to shorter separation paths. The resolution independence of the separation length is a unique feature of IEF and has been verified both experimentally and theoretically. Moreover, due to the specific experimental set-up of MC-IEF, the problematic cross injection, commonly employed for electrophoresis miniaturized to the microchip, could be avoided. The planar arrangement of the microchip also facilitates the implementation of whole-column imaged detection, which was identified as the most suitable detection mode for IEF separation.[40] The successful coupling of MC-IEF to another separation process for 2D separation or to MS clearly demonstrates the potential of MC-IEF technology for system integration. Last but not least, MC-IEF accomplished in cheap plastic microchips shows great promise for the development of disposable chips for routine analyses.

Even though MC-IEF is still at the stage of testing the robustness of the technique for the separation of real protein samples, there is no doubt of its significant impact on

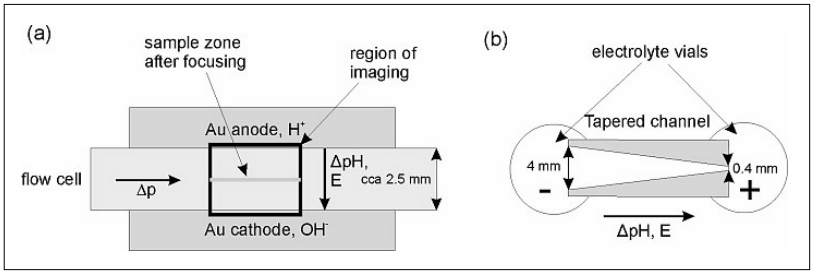


Fig. 3. Alternative ways of pH gradient formation. Schematic drawing of (a) electrically generated pH gradient; (b) thermally generated pH gradient; the arrows indicate the direction of pressure gradient (Δp), electric field (E), and pH gradient (ΔpH).

protein analytics, in particular in the area of proteomics. Due to its high resolving power and spontaneous concentration of proteins, MC-IEF will presumably play an indispensable role in the integrated chip systems for protein analysis. The commercialization of easily operable microfabricated devices employing MC-IEF can be expected in the near future, leading to a revolution in many laboratories for routine analyses, especially in the pharmaceutics, medicine or life sciences.

Acknowledgements:

Partial funding for this project provided by Swiss National Science Foundation, grant number 200020-112190, is gratefully acknowledged.

Received: February 25, 2008

[1] R. Westermeier, 'Electrophoresis in practice', Wiley, Weinheim, **2001**, p. 45.
[2] F. Kilar, *Electrophoresis* **2003**, *24*, 3908.
[3] Q. L. Mao, J. Pawliszyn, *Analyst* **1999**, *124*, 637.
[4] V. Dolnik, S. R. Liu, *J. Sep. Sci.* **2005**, *28*, 1994.
[5] J. Q. Wu, S. C. Li, A. Watson, *J. Chromatogr. A* **1998**, *817*, 163.
[6] B. Yao, H. H. Yang, Q. L. Liang, G. Luo, L. D. Wang, K. N. Ren, Y. D. Gao, Y. M. Wang, Y. Qiu, *Anal. Chem.* **2006**, *78*, 5845.
[7] O. Hofmann, D. P. Che, K. A. Cruickshank, U. R. Muller, *Anal. Chem.* **1999**, *71*, 678.
[8] W. Tan, Z. H. Fan, C. X. Qiu, A. J. Ricco, I. Gibbons, *Electrophoresis* **2002**, *23*, 3638.
[9] J. Han, A. K. Singh, *J. Chromatogr. A* **2004**, *1049*, 205.
[10] C. Das, Z. H. Fan, *Electrophoresis* **2006**, *27*, 3619.
[11] O. Vesterbe, H. Svensson, *Acta Chem. Scand.* **1966**, *20*, 820.
[12] A. V. Stoyanov, C. Das, C. K. Fredrickson, Z. H. Fan, *Electrophoresis* **2005**, *26*, 473.
[13] H. C. Cui, K. Horiuchi, P. Dutta, C. F. Ivory, *Anal. Chem*. **2005**, *77*, 1303.
[14] M. Vlckova, F. Kalman, M. A. Schwarz, *J. Chromatogr. A* **2008**; *1181*, 145–152.
[15] F. Raisi, P. Belgrader, D. A. Borkholder, A. E. Herr, G. J. Kintz, F. Pourhamadi, M. T. Taylor, M. A. Northrup, *Electrophoresis* **2001**, *22*, 2291.
[16] J. C. Sanders, Z. L. Huang, J. P. Landers, *Lab Chip* **2001**, *1*, 167.
[17] C. Das, Z. Xia, A. Stoyanov, Z. H. Fan, *Instrum. Sci.Technol.* **2005**, *33*, 379.
[18] X. Y. Huang, J. C. Ren, *Electrophoresis* **2005**, *26*, 3595.
[19] C. Guillo, J. M. Karlinsey, J. P. Landers, *Lab Chip* **2007**, *7*, 112.
[20] J. Wen, Y. H. Lin, F. Xiang, D. W. Matson, H. R. Udseth, R. D. Smith, *Electrophoresis* **2000**, *21*, 191.
[21] M. L. S. Mok, L. Hua, J. B. C. Phua, M. K. T. Wee, N. S. K. Sze, *Analyst* **2004**, *129*, 109.
[22] M. Fujita, W. Hattori, T. Sano, M. Baba, H. Someya, K. Miyazaki, K. Kamijo, K. Takahashi, H. Kawaura, *J. Chromatogr. A* **2006**, *1111*, 200.
[23] Y. Li, D. L. DeVoe, C. S. Lee, *Electrophoresis* **2003**, *24*, 193.
[24] A. Griebel, S. Rund, F. Schonfeld, W. Dorner, R. Konrad, S. Hardt, *Lab Chip* **2004**, *4*, 18.
[25] Y. Li, J. S. Buch, F. Rosenberger, D. L. DeVoe, C. S. Lee, *Anal. Chem*. **2004**, *76*, 742.
[26] C. Li, Y. N. Yang, H. G. Craighead, K. H. Lee, *Electrophoresis* **2005**, *26*, 1800.
[27] T. M. Huang, J. Pawliszyn, *Electrophoresis* **2002**, *23*, 3504.
[28] S. W. Tsai, M. Loughran, A. Hiratsuka, K. Yano, I. Karube, *Analyst* **2003**, *128*, 237.
[29] A. E. Herr, J. I. Molho, K. A. Drouvalakis, J. C. Mikkelsen, P. J. Utz, J. G. Santiago, T. W. Kenny, *Anal. Chem.* **2003**, *75*, 1180.
[30] Y. C. Wang, M. N. Choi, J. Y. Han, *Anal. Chem.* **2004**, *76*, 4426.
[31] H. C. Cui, K. Horiuchi, P. Dutta, C. F. Ivory, *Anal. Chem*. **2005**, *77*, 7878.
[32] Y. Xu, C. X. Zhang, D. Janasek, A. Manz, *Lab Chip* **2003**, *3*, 224.
[33] D. Kohlheyer, G. A. J. Besselink, S. Schlautmann, R. B. M. Schasfoort, *Lab Chip* **2006**, *6*, 374.
[34] D. Kohlheyer, J. C. T. Eljkel, S. Schlautmann, A. van den Berg, R. B. M. Schasfoort, *Anal. Chem.* **2007**, *79*, 8190.
[35] G. V. Zilberstein, E. M. Baskin, S. Bukshpan, *Electrophoresis* **2003**, *24*, 3735.
[36] G. V. Zilberstein, E. M. Baskin, S. Bukshpan, L. E. Korol, *Electrophoresis* **2004**, *25*, 3643.
[37] G. Zilberstein, L. Korol, S. Bukshpan, E. Baskin, *Proteomics* **2004**, *4*, 2533.
[38] K. Macounova, C. R. Cabrera, P. Yager, *Anal. Chem*. **2001**, *73*, 1627.
[39] K. Macounova, C. R. Cabrera, M. R. Holl, P. Yager, *Anal. Chem*. **2000**, *72*, 3745.
[40] X. Z. Wu, J. Q. Wu, J. Pawliszyn, *LC-GC* **2001**, *19*, 527.