Electrifying the Molecules of Life

Peptide and Protein Analysis by Capillary Electrophoresis Coupled to Electrospray Ionization Mass Spectrometry

BY

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Abstract

This thesis describes the current status and novel aspects of the analysis of the molecules of life, i.e. peptides and proteins, using capillary electrophoresis (CE) coupled to mass spectrometry (MS) via (sheathless) electrospray ionization (ESI). Early reports of sheathless CE-ESI-MS were plagued by limited lifetimes of the electrospray emitter. In this thesis, two new approaches, the Black Dust and the Black Jack methods, utilizing polymer-embedded graphite instead of noble metals are presented. These emitters have shown improved long-term stability and proven excellent for sheathless electrospray operation. Failure of an emitter is often caused by electrochemical reactions occurring at the emitter-liquid interface. The electrochemical properties of the graphite coated emitters were therefore evaluated by classical electrochemical methods, such as cyclic voltammetry and chronoamperometry. The graphite coated emitters showed excellent electrochemical stability and properties compared to noble metal and polymer configurations.

Analyte-wall interactions have long been known to cause problems in the CE analysis of biomolecules. This can be circumvented by internal modification of the capillary walls. Additionally, it is of outermost importance to have a stable and sufficiently high electroosmotic flow (EOF) to sustain the electrospray, when using a sheathless approach. New monomer and polymer coatings are presented for rapid and high-efficient CE-ESI-MS separations of peptides and proteins.

Furthermore, the use of CE-ESI coupled to Fourier transform ion cyclotron resonance mass spectrometry (FTICRMS) shows great potential for rapid proteomic probing of human cerebrospinal fluid. The results are comparable with more established techniques, such as liquid chromatography and two-dimensional gel electrophoresis coupled to MS. However, the CE-ESI-FTICRMS analysis has significantly lower sample consumption and faster analysis time compared to the other techniques. The applications and use of CE-ESI-MS is expected to have a bright future with continued growth as current trends of multidimensional hyphenation and microfabricated devices are further developed and explored.

Keywords: Capillary electrophoresis (CE), Electrospray ionization (ESI), Mass spectrometry (MS), Peptides, Proteins, Cerebrospinal fluid (CSF), Graphite coating, Monomer coating, Polymer coating

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List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals:

I  A simple and robust conductive graphite coating for sheathless electrospray emitters used in capillary electrophoresis/mass spectrometry

II A conductive polymeric material used for nanospray needle and low-flow sheathless electrospray ionization applications

III A comparison of the electrochemical stabilities of metal, polymer and graphite coated nanospray emitters

IV Monomer surface modifications for rapid peptide analysis by capillary electrophoresis and capillary electrochromatography coupled to electrospray ionization-mass spectrometry

V  A polyamine coating for enhanced capillary electrophoresis electrospray ionization mass spectrometry of proteins and peptides

VI Rapid analysis of tryptically digested cerebrospinal fluid using capillary electrophoresis-electrospray ionization-Fourier transform ion cyclotron resonance-mass spectrometry

Permission to reprint the articles was kindly granted by the publishers.
The experimental work, discussion of the results and writing in Paper I was conducted in collaboration with S. Nilsson. I was responsible for planning the experiments in Paper II-IV & VI. The experimental work, discussion and writing in Paper II was done in collaboration with S. Nilsson. In Paper III, O. Klett performed the electrochemical experiments and we wrote the article together. The experimental work, discussion and writing in Paper IV was accomplished together with N. Johannesson. I assisted with the mass spectrometric measurements in Paper V. S. Ullsten and A. Zuberovic planned and performed the other experimental work and wrote the article. M. Palmblad assisted with the experimental work and conducted the data evaluation in Paper VI, while we wrote the article together.

Papers not included in the thesis

- **Analysis of enzymatically digested proteins and protein mixtures using a 9.4 Tesla Fourier transform ion cyclotron mass spectrometer**

- **Optimization of capillary electrophoresis conditions for coupling to a mass spectrometer via a sheathless interface**

- **Effect of sequence length, sequence frequency, and data acquisition rate on the performance of a Hadamard transform time-of-flight mass spectrometer**

- **Hadamard transform time-of-flight mass spectrometry: A high-speed detector for capillary-format separations**
- Peptide mapping of proteins in human body fluids using electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry

- Capillary electrophoresis and electron capture dissociation Fourier transform ion cyclotron resonance mass spectrometry for peptide mixture and protein digest analysis

- On-column polymer-imbedded graphite inlet electrode for capillary electrophoresis coupled on-line with flow injection analysis in a poly(dimethylsiloxane) interface

- Comparison between different sheathless electrospray emitter configurations regarding the performance of nanoscale liquid chromatography time-of-flight mass spectrometry analysis
## Contents

1 Introduction ................................................................................................. 1

2 Capillary electrophoresis ........................................................................ 3
  2.1 Basic concepts of CE ........................................................................... 3
    2.1.1 CE instrumentation ...................................................................... 4
  2.1.2 Electrophoretic migration and electroosmosis ............................... 5
    2.1.3 Sample injection ......................................................................... 7

3 Electrospray ionization ............................................................................. 9
  3.1 The ESI process ............................................................................... 9
    3.1.1 Electrochemical aspects of ESI .................................................. 12
  3.2 Interfacing CE and ESI-MS ............................................................. 13
    3.3 Nanoelectrospray ......................................................................... 17

4 Mass spectrometry .................................................................................. 19
  4.1 CE-ESI-MS ................................................................................ 19
    4.1.1 CE-ESI-TOFMS ...................................................................... 20
    4.1.2 CE-ESI-FTICRMS .............................................................. 21

5 Tailored surfaces for CE-ESI-MS ............................................................. 23
  5.1 Positively charged surfaces ........................................................... 23

6 Analyzing biological samples ................................................................. 27

7 Future aspects ....................................................................................... 32

8 Concluding remarks ............................................................................. 34

9 Acknowledgements ............................................................................. 35

10 Swedish summary ............................................................................... 36

11 References .......................................................................................... 39
Abbreviations

APS 3-aminopropyltriethoxysilane
CE Capillary electrophoresis
CRM Charge Residue Model
CSF Cerebrospinal fluid
EOF Electroosmotic flow
ESI Electrospray ionization
FAB Fast atom bombardment
FTICR Fourier transform ion cyclotron resonance
IEM Ion Evaporation Model
LC Liquid chromatography
LIF Laser induced fluorescence
MALDI Matrix assisted laser desorption ionization
MAPTAC [3-(methacryloylamino)propyl]trimethylammonium chloride
MS Mass spectrometry
m/z Mass-to-charge ratio
ODAC Octadecyldimethyl(3-trimethoxysilylpropyl)ammonium chloride
PAGE Polyacrylamide gel electrophoresis
SPE Solid phase extraction
TAC N-Trimethoxysilylpropyl-N,N,N-trimethylammonium chloride
TeDAC Tetradecyldimethyl(3-trimethoxysilylpropyl)ammonium chloride
TOF Time-of-flight
T Tesla
UV-vis Ultraviolet-visible
1 Introduction

With the unraveling of the human genes in the Human Genome Project (HUGO), focus of biologists, biochemists, chemists and physicists has once again turned to proteins and peptides. The genome is the blueprint of the cell, whereas proteins and peptides are the building blocks and actors both within and outside the cell. In order to understand the complexity of studying the protein expression within a particular cell or tissue (Proteomics) one must consider a number of factors. First, each gene in a cell may, by different splicing patterns, give rise to a number of proteins. After translation, the formed proteins may be additionally modified by e.g. enzymatic cleavage, glycosylation or phosphorylation. The collective term for such changes is posttranslational modification. These modifications imply that a cell with 10 000 genes may contain more than 50 000 different proteins. Second, the proteins within a cell are not homogenously distributed throughout the cell. Proteins exist in defined sub-cellular compartments (organelles) such as nucleus, mitochondria, membrane and cytoplasm and are able to move between these compartments, causing a great variation of both composition and concentration within the cell. Finally, in contrast to the genome, the proteome is constantly changing. Protein expression, modification and location are dynamic processes that vary depending on the needs of a cell. Taking these factors into consideration, one realizes that the demands on the analytical tools for proteomic research are enormous. There is a constant need to develop new and refined sampling, separation, detection and data handling techniques.

The three key approaches to purify and separate proteins are ultracentrifugation, electrophoresis and chromatography. Swedish scientists have contributed with pioneering discoveries and developments in these areas. Theodor (The) Svedberg, awarded the Nobel Prize in chemistry 1926, was one of the developers of the ultracentrifuge, allowing analysis of large molecules such as proteins and polymers in solution [1]. Following in Svedberg’s footsteps, Arne Tiselius refined and developed electrophoretic and adsorption (chromatographic) techniques for the analysis of proteins in biological fluids [2]. For his contributions in these fields, Tiselius was awarded the Nobel Prize in chemistry in 1948. One of Tiselius students, Stellan Hjertén, further developed electrophoresis in describing free zone electrophoresis [3], in which rapid and efficient separations of ionic species and macromolecules were performed in rotating quartz capillaries. During
the 40s to the 70s variants of electrophoresis, e.g. isoelectrofocusing [4] and polyacrylamide gel supported electrophoresis [5] were developed. These techniques are now routine analytical techniques in the proteomic/biomedical laboratories. During the late 70s and early 80s, the advantages of using narrow inner diameter (i.d.) capillary tubing were reported [6, 7]. Today, capillary electrophoresis (CE) utilizing fused silica capillaries with i.d. less than 100 µm is a well established analytical tool.

Mass spectrometry (MS) is a very powerful technique to measure mass-to-charge ratios of chemical species. Although MS has been used for more than a century, it was not until the introduction of fast atom bombardment [8] and more importantly electrospray ionization (ESI) [9] and matrix assisted laser desorption ionization [10] that MS led to major breakthroughs in biological and biochemical research. Capillary electrophoresis was first coupled to ESI-MS fifteen years ago [11]. This coupling has since grown to be a continuously more important analytical technique.

This thesis deals with the current status, improvements and considerations of CE-ESI-MS, especially for the analysis of peptides and proteins. Furthermore, the use of this technique in clinical studies of complex biological samples, such as human body fluids is shown. Finally, future aspects and trends of CE-ESI-MS are discussed.
2 Capillary electrophoresis

Electrophoresis is defined as the migration of ions in a solution or gel under the influence of an electric field. The direction and velocity of an ion's electrophoretic migration is dependent on its charge and size, as described in section 2.1.2. In capillary electrophoresis, the separation of the analytes takes place in capillaries with a small inner diameter (usually < 100 μm). This yields high-resolution separations with very high efficiencies (up to 1000 000 theoretical plates or more). CE, and variations thereof, have been employed for a wide range of analytes including small molecules such as inorganic ions, organic acids, amino acids, peptides, drugs, nucleosides, nucleotides, vitamins, steroids and carbohydrates as well as large molecules e.g. hormones, proteins, nucleic acids and even living cells. For further aspects and applications of CE and related techniques, a number of review articles [12-20] and books [21, 22] are recommended.

2.1 Basic concepts of CE

The separation efficiency (N) of CE, as well as chromatography, is based on the Van Deemter equation (1), in which the plate height, \( H \), is related to the velocity, \( v_x \), of the carrier liquid along the separation axis, \( x \).

\[
H = A v_x + \frac{B}{v_x} + C v_x
\]  

(1)

\( A, B \) and \( C \) are constants, corresponding to a flow dispersion term, longitudinal diffusion term and mass-transfer term respectively. Since CE separations are carried out in open capillaries (due to absence of packing material as commonly used in chromatography), the constants \( A \) and \( C \) can be eliminated in equation (1), leaving only longitudinal diffusion contributions to effect the plate height. Furthermore, the longitudinal diffusion contributions in CE are rather small due to fast separation times. The small value of \( H \) implies that a large number of theoretical plates, \( N \), are given for a certain length along the separation axis of CE. In addition, the resolution between two analytes in the separation is proportional to \( N^{1/2} \).
Thus, CE yields high-resolution and very high efficiencies. With this given, the separation times can be reduced to the order of seconds.

2.1.1 CE instrumentation

The basic CE instrumentation is simple and inexpensive. It consists of two buffer vials, a fused silica capillary, a high voltage power supply, two electrodes, a detector and a data collection and analysis system, which is usually a computer. Figure 1 shows a principal CE set-up.

\[ \text{Figure 1. The principal instrumental set-up for capillary electrophoresis. The enlargement shows the direction of the electrophoretic mobilities of the ions and the electroosmotic flow.} \]

The sample is usually injected (section 2.1.3) at the anodic end of the capillary and a high potential difference (typically 10-30 kV) is applied between the two buffer vials, which in turn causes electrophoretic and electroosmotic movement of ions in the capillary. UV-vis is the most common detection technique. However, various techniques such as laser induced fluorescence (LIF) [23], electrochemical detection [24] and mass spectrometry (MS) [25, 26] can be employed as means of detection. This thesis focuses on the on-line combination of CE and MS, using electrospray ionization.
2.1.2 Electrophoretic migration and electroosmosis

Under the influence of the applied electric field, an ion in the capillary will experience a force driving it towards its counter electrode, hence causing electrophoretic migration. This force \( F_e \) is proportional to the ion's charge \( q \) and the electric field strength \( E \). The driving force is counteracted by a frictional force \( F_f \), which is proportional to the ion velocity \( v_e \) and the friction coefficient \( f \). As a consequence, the ion will almost immediately reach a steady state, where the opposing forces are balanced (2).

\[
qE = f v_e
\]  
(2)

The steady state velocity of an ion can thereby be determined (3).

\[
v_e = \frac{q}{f} E = \mu_e E
\]  
(3)

Where \( \mu_e \) is the electrophoretic mobility of the ion. The electrophoretic mobility of an ion is proportional to its charge and inversely proportional to the friction coefficient. The friction coefficient of a spherically hydrated ion is given by equation (4).

\[
f = 6\pi \eta r
\]  
(4)

Where \( \eta \) is the viscosity of the solution and \( r \) is the hydrated radius of the ion. From equation (3) and (4), one can see that the electrophoretic mobility of an ion is dependent on its charge and size. Small, highly-charged ions will have the largest electrophoretic mobility. Notice, however, that this electrophoretic force has no effect on neutral analytes.

There is a solvent flow in CE, caused by the charged silica surface of the capillary and the buffer ions. This is called the electroosmotic flow (EOF). The silica surface contains protolytic silanol groups (Si-OH) with a pK_a value of \( \approx 3-6 \). As a result, using buffers with a pH > 3 yield a negatively charged surface. The negative charge on the surface is counter balanced by positive ions in the buffer, forming an electric double layer (Figure 2). According to the Goy-Chapman-Stern model, this electric double layer consists of a static inner Helmholtz, or Stern layer and an outer diffuse layer. When the electric field is applied over the capillary, the hydrated, positively charged ions in the diffuse layer will start to migrate towards the cathode. The movement of the diffuse layer is propagated through the buffer due to viscous forces, thus pulling the buffer and all analytes through the capillary.
Figure 2. Schematic figure of the electric double layer in CE.

The velocity ($v_{eo}$) of the EOF is given by equation (5).

$$v_{eo} = \mu_{eo} E \quad (5)$$

Where $\mu_{eo}$ is the electroosmotic mobility. The electroosmotic mobility is in turn given by the Helmholtz-Smoluchowski equation (6).

$$\mu_{eo} = \frac{\varepsilon \zeta}{4\pi \eta} \quad (6)$$

Where $\varepsilon$ is the permittivity of the solution and $\zeta$ is the zeta potential over the double layer. As can be seen in Figure 2, the zeta potential is decreasing over the double layer until it reaches a constant value outside the diffuse layer. The size of the diffuse layer is small compared to the bulk solution in the capillary. This yields a plug shaped flow of the EOF, which is one of the sources for the high efficiencies obtained in CE. The volumetric flow in CE is typically on the order of a couple of hundred nL/min.

The measured, or apparent, mobility of an ion in a CE separation is the sum of the electrophoretic and electroosmotic mobilities (7).

$$\mu_{app} = \mu_e + \mu_{eo} \quad (7)$$
At moderate pH values, the electroosmotic mobility tends to be larger than the electrophoretic mobility, yielding a positive mobility of all ions. In other words, the ions are separated based on their electrophoretic mobility, while the EOF drives all analytes towards the detector. As further discussed in section 3.2 and 5, it is important to control the surface of the capillary, and thereby the EOF in order to yield robust CE separations in combination with mass spectrometry. This is especially important for analytes that have a tendency to interact with the fused silica surface, i.e. peptides and proteins.

2.1.3 Sample injection
The sample volume injected in CE is often very small, typically a few nanoliters or less. The small injection volume can be considered advantageous when the sample volume is limited. However, it is also a disadvantage, as only a restricted amount of sample can be injected. A general rule of thumb is that the injection volume should not exceed 2-4 % of the total capillary volume [22]. Injection volumes that are too large tend to yield separations with reduced efficiency and resolution. The two most common injection techniques are hydrodynamic and electrokinetic injection.

Hydrodynamic injection is performed by applying a pressure difference ($\Delta P$) between the capillary ends. The injected volume ($V_i$) can be calculated by the Poiseuille equation (8).

$$V_i = \frac{\Delta P \pi d^4 t}{128 \eta L_c}$$  (8)

Where $d$ is the inner diameter of the capillary, $t$ is the injection time, $\eta$ is the sample viscosity and $L_c$ is the total length of the capillary. Electrokinetic injection is performed by applying an electric potential (typically a few kV) across the capillary for a fixed time, with the injection end of the capillary immersed into the sample. The number of moles of each analyte that is injected ($Q_i$) is dependent on the apparent velocity ($v_{app}$) of the analyte, the conductivity ratio of the separation buffer and sample ($k_b/k_s$) and the injection time as given in equation (9).

$$Q_i = v_{app} \frac{k_b}{k_s} \frac{t}{r^2} C_i$$  (9)

Where $r$ is the capillary radius and $C_i$ is the concentration of the analyte. Both injection techniques have their advantages and disadvantages. There is no sample bias in the hydrodynamic injection as cations, neutrals and anions are mutually injected and the amount of each analyte injected is independent
of the electrophoretic mobilities. However, too large of a pressure difference may invoke a parabolic plug profile, which can lead to band broadening in the separation. The electrokinetic injection is selective, since the amount of each analyte injected is dependent on the magnitude and direction of their electrophoretic mobilities.

Because CE sample volumes are inherently limited, several sample pre-concentration techniques have been utilized for CE [27-29]. The simplest form of pre-concentration is field amplified sample stacking [6, 30, 31]. In this technique, the analytes are dissolved in a solution with a lower conductivity than the separation buffer. A lower conductivity will yield a higher electric field over the injection plug compared to the buffer. The increased field implies that the sample ions will have a higher velocity through the sample plug. As the ions reach the boundary between sample solution and buffer their velocity will decrease due to the lower field strength. Hence, the ions will stack in narrow bands at the buffer interface. Cations and anions will stack at opposite sides of the injection plug, while neutrals, as aforementioned, are unaffected by the imposed field. Too large of a difference in ionic strength (more than 10 times) between buffer and sample is not recommended as it may cause band broadening [30, 31]. Furthermore, a high conductivity difference may have implications in the electrospray process (see section 3). Apart from on-line pre-concentration, various off-line techniques, such as solid phase extraction (SPE) can be used prior to injection. This is especially useful when the sample contains high amounts of inorganic salts or other contaminants that, if not removed, will affect the separation performance and the mass spectrometric analysis. Off-line SPE sample handling of enzymatically digested proteins and protein mixtures were performed prior to CE-MS analysis in Paper IV-VI.
3 Electrospray ionization

The phenomenon of electrospray has been known for at least two centuries. However, it was not until the late 1960s and early 70s that Dole reported the use of electrospray for the ionization and measurement of macro-ions [32, 33]. In the early 80s, Yamashita and Fenn [9] employed electrospray for the first time to generate gas phase ions for mass spectrometric measurements. Since then, electrospray ionization (ESI) has become one of the most utilized ionization techniques for mass spectrometry (MS).

ESI is a method of transferring ions in a liquid to the gas phase. This is important because a lot of the separation techniques used today (such as LC and CE) are conducted in a solution, whereas the MS operates under vacuum. ESI is a rather straightforward technique that usually is performed at ambient pressure and temperature. It is considered a soft ionization technique, in the sense that it yields essentially no fragmentation. This, together with the ability to spray pure water solutions, is especially important for the MS analysis of intact proteins and protein-protein interactions. However, the most prominent feature of ESI is multiple charging of large analytes [34, 35]. Multiple charging of a large analyte brings its mass-to-charge ($m/z$) ratio down to the working range of the mass spectrometer, facilitating MS analysis of proteins and protein complexes with a molecular weight as large as 3.3M Dalton [36]. Yet, multiple charging yields a more complex mass spectrum as the analyte signal is spread out on numerous peaks, which also reduces the sensitivity.

3.1 The ESI process

ESI is produced by applying a potential (usually a few kV) difference between a hollow emitter and a counter electrode. The electric field will cause electrophoretic movement of ions in the liquid at the tip of the capillary. If a positive potential is applied on the tip, the cations will migrate towards the surface of the liquid whereas the anions will move inwards the capillary, as shown in Figure 3.
When the mutual repulsion between the cations at the surface and their electrostatic attraction towards the counter electrode overcome the surface tension, the surface of the liquid elongates into a Taylor cone [37, 38]. At a certain potential (onset voltage), the electric field exceeds the surface tension holding the surface together and the Taylor cone breaks up into a liquid jet, which in turn splits into small positively charged droplets. The onset voltage ($V_{on}$) can be estimated with equation (10).

\[
V_{on} \approx \left( \frac{r_c \gamma \cos \theta}{2 \varepsilon_0} \right)^{1/2} \ln(4d / r_c) \tag{10}
\]

Where $r_c$ is the outer radius of the capillary or tip, $\gamma$ is the surface tension of the solvent, $\theta$ is the half angle of the Taylor cone, $\varepsilon_0$ is the permittivity of vacuum and $d$ is the distance between tip and counter electrode. Solvent evaporates from the charged droplets as they travel towards the counter electrode, while the charge remains constant, causing an increase in the charge-to-surface ratio. This process will undergo until conditions near the Raleigh stability limit are reached. The Raleigh stability limit is defined as the limit of surface charge density ($q_R$), where the electrostatic repulsion at the surface overcome the surface tension holding the droplet together (11).
\[ q_{Ry} = 8\pi(\varepsilon_0yR^3)^{1/2} \]  \hspace{1cm} (11)

Where \( R \) is the radius of the droplet. At conditions near the Raleigh stability limit (0.7-0.8 \( q_{Ry} \)), the droplets undergo uneven fission, emitting smaller highly charged droplets \([39, 40]\). The uneven fission yields offspring droplets with roughly 1/10 of the radius, 2% of the mass and 15% of the charge of the parent droplets \([39]\). Thus, the charge-to-surface ratio increases in each droplet fission event. The offspring droplets will in turn undergo uneven fission, giving rise to new offspring droplets. This process of uneven fission will progress until gas phase ions are produced.

The mechanism for the production of gas phase ions is still not completely known and under debate \([41-43]\). There are two dominating theories, the Charge Residue Model (CRM) and the Ion Evaporation Model (IEM). The schematics of the CRM and the IEM are given in Figure 4.

\[ \text{Figure 4. Schematics for the two dominating theories, the Charge Residue Model (CRM) and the Ion Evaporation Model (IEM), for the generation of gas phase ions in ESI.} \]

The CRM, initially proposed by Dole \([32]\), suggests successive droplet fission leading to droplets containing only one ion. This molecule becomes a free gas phase ion as the last of the solvent evaporates. The IEM, launched by Iribarne and Thompson \([44]\), also assumes repeated droplet fission. However, when the radius of the droplets subsides 10 nm, the field on its surface becomes strong enough to overcome solvation forces and the ions...
are ejected from the droplet surface into the gas phase. It is not yet known which of these models is the predominant mechanism for the generation of gas phase ions. The current belief is that the CRM is the most likely mechanism for the generation of gas phase macro-ions [45], while the IEM probably dominates for small, surface-active ions. Both mechanisms can though occur simultaneously, depending on the analyte.

3.1.1 Electrochemical aspects of ESI

The ESI process can be described as a controlled current electrochemical flow cell [43, 46-48], where the current is sustained by a number of electrochemical reactions occurring at the emitter tip and counter electrode. The ESI current \( i_{ES} \) has been found to be dependent on several parameters [47, 49], as given in equation (12).

\[
i_{ES} = H v_f \sigma_s^n E_c^v
\]  

Where \( H \) is a constant whose value will vary depending on the permittivity and surface tension of the solvent, \( v_f \) is the volumetric flow rate, \( \sigma_s \) is the specific conductivity of the solution and \( E_c \) is the imposed electric field at the capillary tip. The exponents, \( v, n \) and \( \varepsilon \), are interrelated (all > 0) and vary with the parameters.

The electrospray current is carried by the charged droplets, which result in a continuous steady state current. In positive ESI mode, positively charged droplets continuously leave the tip in order to maintain the current. Redox reactions (oxidations at the tip and reductions at the counter electrode) must occur to maintain the charge balance in the cell. Depending on the electrospray current, the nature of the sprayed solution and the ESI interface design, the oxidation reaction (or reactions) with the lowest redox potential will occur. Increasing the electrospray current by increasing the volumetric flow rate, specific conductivity of the solvent or applied electric field (equation 12), will eventually increase the potential at the tip to the redox potential of additional electrochemically active species present in the solution or to the redox potential for the material mediating the ESI voltage. Thus, increasing the current may force additional redox reactions to occur. The solvent used in ESI experiments usually contains some water. The reaction for the oxidation of water is given by equation (13).

\[
2 \text{H}_2\text{O} (l) \rightarrow 4e^- + 4\text{H}^+ (l) + \text{O}_2(g) \quad +1.23 \text{ V vs. NHE}
\]  

In most cases, the concentration of water is sufficient to maintain the ESI current and as a result, the oxidation of water is the potential limiting reaction at the tip [43]. Protons are formed in this reaction, which yields a
reduced pH in the sprayed droplets [48]. Furthermore, the formation of oxygen can have implications in both the ESI process and a combined CE separation. Gas formation can decrease the stability of the spray and cause loss of electrical contact for the CE circuit. It will also put mechanical stress on the conductive layer used to mediate the ESI voltage, as described in section 3.2. One should also be aware of the fact that the oxidation potential for the material used to mediate the ESI voltage to the sprayed liquid is in the vicinity of the oxidation potential for water. Thus, the material will also be oxidized, causing electrochemical stress on the material. Mechanical and electrochemical stress are two limiting factors for the lifetime of the electrospray emitter.

The fact that ESI behaves as a controlled current electrochemical cell can be taken advantage of. Traditional electrochemical experiments, such as cyclic voltammetry, chronopotentiometry and chronoamperometry can easily be conducted on the electrospray emitters to evaluate their electrochemical properties and stability [50, 51]. These experiments can be used as guidelines when operating in electrospray mode. In Paper I and III, such experiments were performed to investigate the performance of electrospray and nanoelectrospray emitters (see section 3.2 and 3.3). Further, the ongoing redox processes in ESI can be studied per se [52].

3.2 Interfacing CE and ESI-MS

Interfacing CE and ESI-MS is not always straightforward. One cannot simply apply a CE method developed for e.g. UV detection to ESI-MS. The buffer composition must be compatible with both the ESI process and MS analysis [53, 54]. The buffers used for CE-ESI-MS should preferably contain volatile buffer ions, such as acetate or formate. The ionic strength should be rather low and the addition of an organic modifier is known to improve the ionization efficiency. Furthermore, a stable and sufficient EOF is often required. Several parameters regarding the ESI interface needs to be considered. An ideal ESI interface should:

- Have minimized band broadening effects on the separation
- Be sensitive, accurate and yield linear response
- Close the electrical circuit for CE
- Handle all species delivered by the CE
- Be compatible with the flow rates generated by the CE
- Be robust over time and variations in sample composition
Three different approaches, the sheath flow interface [55], the liquid junction interface [56] and the sheathless interface [11] (Figure 5) are commonly used for the on-line combination of CE-ESI-MS.

Figure 5. The three different CE-ESI-MS interfaces utilized. The top figure shows the sheath flow interface, the middle shows the liquid junction interface and the bottom figure shows the sheathless interface.

The sheath flow interface is the most widely employed interface [26]. In this set-up, the CE outlet is introduced through a narrow metal tube, which delivers a sheath liquid to the end of the separation capillary at a constant
rate. The mixing of separation and sheath liquid provides the electrical contact between the CE buffer and the ESI needle and closes the CE circuit. An additional sheath gas may sometimes be added to assist the electrospray formation. The flow rate of the sheath liquid is typically in the µL/min range, whereas the EOF is in the nL/min range. This implies that the sheath liquid, due to the higher flow rate, dominates the ESI process, which gives a higher freedom regarding the choice of CE buffer. Less volatile buffer ions and higher ionic strengths may be used. Furthermore, the addition of a sheath liquid often increases the stability of the ESI process. However, adding a sheath liquid may increase the background noise [57], may cause shifts in the migration order of the analytes [58] and will dilute the eluting analyte concentrations, thus causing lower sensitivity and sometimes ion suppression of the analyte ions.

In the liquid junction interface, a liquid reservoir surrounding the junction of the separation capillary and a transfer capillary provides the ESI contact. The separation capillary and the inlet end of a ESI needle are positioned opposite each other with a gap of 10-20 µm, allowing make up liquid from the reservoir to be drawn into the ESI needle. The liquid junction interface is not commonly used due to the difficulty of reproducibly aligning the capillaries and due to many of the same drawbacks described for the sheath flow interface.

In the sheathless interface, a conductive coating is applied on the outlet end of the capillary or the outlet end is inserted into an electrospray emitter (a stainless steel needle, for instance), thus providing the electrical contact between the separation buffer and the ESI potential. When a conductive coating is applied, the outlet end is usually tapered to improve the ESI performance [38]. The tapering of the capillary can be accomplished chemically [57], thermally [59] or mechanically [60, 61]. The conductive coating on the tapered tip usually consists of gold [59, 61-65] or silver [66, 67]. Various methods for applying the metal layer on the tip have been described. These include: electroplating, evaporating, sputtering and gluing [59, 61-66, 68, 69]. With a few exceptions [63, 65], these metal coatings have short lifetime (less than 100 h) that limit their range of application. Graphite is an attractive alternative to metals for the use in the conductive coatings [70-72]. In Paper I and II, two different graphite coatings, the Black Dust and the Black Jack method, are described. The Black Dust coating consists of a mixture of polyimide and graphite (see Figure 6). This mixture can be easily applied to the exterior of fused silica capillaries and the coating has proven excellent for electrospray operation. Among its desirable features are long lifetime (more than 300 h), good electrochemical resistance and minimized band broadening effects on a separation. The Black Dust method has been implemented for nanoelectrospray (section 3.3), CE (Paper I) and nanoscale LC experiments [73]. Furthermore, it has also been used successfully as an on-column integrated inlet electrode [74, 75]. The Black
Jack coating (Figure 6) consists of a mixture of polypropylene and graphite, which can also be applied on silica capillaries. However, the major benefits of this emitter are in nanoelectrospray mode, where the entire emitter consists of this mixture.

Figure 6. Pictures of the graphite based electrospray and nanoelectrospray emitters presented in Paper I and II. The top left picture shows the Black Dust emitter, the top right shows the Black Jack coating applied on fused silica capillary and the two bottom pictures show the Black Jack nanoelectrospray emitter.

Another alternative for sheathless ESI is the “up stream” interface, where a small hole is drilled near the outlet end of the capillary. Electrical contact for the ESI is provided by covering the hole with gold epoxy [76] or by inserting a small platinum electrode through the hole [77]. However, electrochemical reactions occurring at the ESI contact, as described in section 3.1.1, may influence the overall performance.

The sheathless interface places strict limitations on the buffer composition [49, 54, 78], as more volatile buffer ions and the addition of an organic modifier are preferred to improve the ESI. Furthermore, a stable EOF is also necessary to sustain the electrospray and the interface usually has a limited lifetime. The advantages of a sheathless interface are higher sensitivity, due to no dilution of the eluting analytes and minimized band broadening on the separation.
3.3 Nanoelectrospray

Nanoelectrospray [38, 79] or microelectrospray [80, 81] are based on further downscaling of the sheathless electrospray interface. Nanoelectrospray (Nanospray) is usually performed in pulled capillaries with an inner diameter of a few µm. The applied electric field for the electrospray process induces the sample solution flow, typically in the 10-20 nL/min range. Additional pumps or liquid driving forces are not usually necessary. The reduced flow rates and emitter dimensions yield droplets that are smaller than those in conventional ESI [82]. Small droplets have a high surface-to-volume ratio, which yields a number of desirable features including increased sensitivity and a higher tolerance to salts in the samples [83]. Additionally, the sample consumption is very low compared to conventional electrospray. Thus, nanospray is especially beneficial when sample volumes and analyte concentrations are limited.

The tip of the emitter is coated with methods and materials (often metals) similar to the sheathless electrospray emitters. There have also been reports of using conductive polymers, such as polyaniline (PANI) [84-86] or graphite coatings [87]. However, most of these emitters have shown lifetimes of a couple of hours, restricting their use. In Paper II, the production and performance of Black Jack nanospray emitters is described (See Figure 6). These emitters, which consisted entirely of the polypropylene/graphite mixture, showed good long-term stability (at least 24 h), as well as excellent mechanical and electrochemical stability. The electrochemical properties of metal, polymer and graphite coated nanospray emitters were evaluated in Paper III. Based on cyclic voltammetry and chronoamperometric tests (Figure 7), it was concluded that the loss of electrochemical activity for the metal coated emitters was due to loss of conductive coating, while the Black Dust emitters were only passivated. The Black Jack emitters were unaffected by the electrochemical tests and the PANI coated emitters could not be thoroughly investigated due to limited electrochemical activity.
Figure 7. Chronoamperometric current transients of the different nanoelectrospray emitters investigated in Paper III. The Black Dust (1) and Black Jack (5) coatings contain graphite, while the New Objective (2), WPI (3) and Protana (4) coatings consisted of a noble metal. The PANI coated emitter could not be tested by chronoamperometry. It should be noted that y-axis of the curves in Figure 3 in Paper III also should be denoted $i/i_{avg}$ (current/average current).

Although benefits of using pure nanospray have been shown, its future lies in the combination with separation techniques and especially as an integrated emitter in microfabricated devices (section 7). This implies cheap but robust emitters with good long-term stability, as shown for the graphite coated emitters.
4 Mass spectrometry

A mass spectrometer (MS) separates and measures charged species (ions) in the gas phase. The separation of ions is based on their mass-to-charge ratio and is conducted by applying electric or magnetic fields. Although the modes of operation may vary between different MS instruments, all instruments have, in principle, the same components: vacuum pumps, an ion source, ion optics, a mass analyzer, a detector and a data handling system as shown in Figure 8.

![Figure 8. General components of a MS instrument with a reflectron time-of-flight mass analyzer.](image)

4.1 CE-ESI-MS

CE-ESI has been coupled on-line to MS using various mass analyzers including magnetic sector [88], quadrupole [11], ion trap [89, 90], time-of-flight (TOF) [91] and Fourier transform ion cyclotron resonance (FTICR) [92] instruments. The quadrupole and ion trap instruments are the most commonly used for CE-ESI-MS [26]. This merely reflects current laboratory
equipment status as quadrupole instruments are the most widespread and ion traps are relatively inexpensive. In this thesis, TOFMS and FTICRMS instruments have been explored as interesting alternatives. The TOFMS is a fast acquiring and reasonably inexpensive instrument with, in theory, unlimited m/z range. The coupling of CE-ESI-TOFMS is further described in section 4.1.1. The major benefit of the FTICRMS instrument is its unsurpassed mass resolving power and high mass accuracy, as described in section 4.1.2 and section 6.

4.1.1 CE-ESI-TOFMS

In TOFMS, ions are accelerated by an electric field and their flight times over a certain distance are measured. Low m/z ions fly faster than high m/z ions and are thus separated. Conventional time-of-flight techniques with a continuous ion source, such as ESI, are almost exclusively based on orthogonal extraction (OE) in which the ions are pulsed at a right angle towards the detector.


The duty cycle of the OE-TOFMS is related to the repetition rate. The repetition rate is dependent of the duration of a single mass spectrum, which
in turn is determined by the time for the heaviest ions to reach the detector. The repetition rate is usually on the order of kHz. However, several spectra are usually bunched together, giving an acquisition rate on the order of spectra/second. Available instruments with an acquisition rate of up to 100 spectra/second are fully adequate for CE-ESI-MS applications, as shown in Figure 9. A fast acquiring OE-TOFMS instrument was used in Paper I-V.

One alternative TOFMS technique is the linear Hadamard transform TOFMS [93, 94]. In this technique, the continuous ion beam is constantly split into discrete ion packets of different lengths following a pseudorandom sequence (PRS) of pulses based on Hadamard type binary sequences. As the ions are constantly reaching the detector, the overall signal will be an overlap of many time-of-flight distributions, each one shifted following the pattern dictated by the PRS. The obtained overlapping raw spectrum is deconvoluted using a fast Hadamard transform algorithm, with the same sequence applied to split the beam, yielding a conventional TOF mass spectrum. The benefits of the Hadamard transform TOFMS are high duty cycle and ion transmission. Additionally, an acquisition rate of more than 250 spectra/second has been reported [95]. These features are potentially beneficial for the on-line coupling of CE-ESI-MS.

4.1.2 CE-ESI-FTICRMS

The basis of FTICRMS is the movement of ions in a strong magnetic field. The ions are trapped inside a cell of the instrument, where a strong unidirectional magnetic field (usually several Tesla) is superimposed. Under the influence of the magnetic field, the ions will start to move in a circular motion perpendicular to the field. This motion is called the ion cyclotron motion and is characterized by the cyclotron frequency, which is how often an ion repeats its orbit. The cyclotron frequency, \( f_c \), is dependent on three parameters, the strength of the magnetic field, \( B \), the charge of an ion, \( z \), and the mass of an ion, \( m \), as shown by equation (14).

\[
 f_c = \frac{zB}{2\pi m} \quad (14)
\]

Usually, the magnetic field is constant and the mass-to-charge ratio is determined by measuring the cyclotron frequency. It should be noted (from equation 14) that the cyclotron frequency is independent of an ion's velocity or kinetic energy. Increasing an ion's velocity will merely increase the radius of its circular motion. This is taken advantage of for the detection of the ions, where a radio frequency electric field is applied for excitation and detection of the ions. The independence of the cyclotron frequency from the kinetic energy of an ion, together with the high vacuum in the cell, the long
transient acquisition times (sometimes hours!) and the coherent excitation and detection of ions with the same m/z are factors responsible for the high mass resolution and mass accuracy in FTICRMS. However, when coupling a fast separation technique (e.g. CE) to the FTICRMS, the transient acquisition times are limited to durations compatible with the peak widths of the separation. The acquisition rate is restricted by a fixed series of events in the cell and is at the most on the order of tenths of a second. This can have implications when combining rapid CE separations with FTICRMS, where the CE peak widths can be less than a second. Nonetheless, CE has successfully been coupled to FTICRMS [92], especially for the analysis of complex samples [96-98]. Aside from the benefit of added chemical information, space charge effects in the cell and ion suppression effects are decreased when a separation technique is introduced. In addition, MS fragmentation techniques, such as sustained off resonance irradiation (SORI) and electron capture dissociation (ECD) have been implemented in the analysis [99, 100]. CE-FTICRMS analysis of a complex human body fluid is described in Paper VI and also further discussed in section 6.
5. Tailored surfaces for CE-ESI-MS

Unmodified walls of fused silica capillaries are simple and, in many cases, fully adequate surfaces for CE applications. However, the protolytic silanol groups (Si-O-H) on the surface may sometimes negatively affect the analysis. There are several reasons why a coating of the surface may be advantageous. The EOF tend to be unstable in bare fused silica capillaries, leading to shifts in the migration times of the analytes. Furthermore, when using a sheathless ESI interface in CE-MS, the EOF must be high and stable enough to sustain the electrospray (as mentioned in section 3.2). Peptides and proteins are often the analytes of interest and analyte-wall interaction has long been known to be a problem in these analyses. Especially proteins and basic peptides show strong electrostatic interaction with the negatively charged surface. Additionally, hydrophobic interactions may also occur. These analyte-well interactions lead to band broadening, irreproducible migration times, irregularities in the EOF and, in worst case, total loss of the analytes. It is worth noting that controlled analyte-wall interactions can be utilized for its selectivity, as for instance in open tubular capillary electrochromatography (OT-CEC) [101], where an added chromatographic dimension allows complementary increased resolution of specific analytes and efficient separation of neutrals.

Numerous approaches have been investigated to control analyte-wall interactions, including: extreme pH buffers [102], high salt concentrations [103], various buffer additives [104, 105] and capillary surface coatings [106, 107]. When using MS in combination with ESI, robust surface coatings are preferred because high ionic strengths as well as non-volatile buffer additives may interfere with the ESI process and bias or even spoil the MS analysis [108]. Furthermore, extreme pH buffers can denaturate or precipitate analytes such as proteins. Various monomer and polymer coating procedures yielding a neutral, negative or positive surface have been reported [106, 107]. However, this thesis focuses on positively charged surfaces suitable for CE in combination with sheathless ESI-MS.

5.1 Positively charged surfaces

The major benefit of using positively charged inner surfaces is the suppression of electrostatic analyte-wall interactions for basic peptides and
proteins. Moreover, an EOF that is high and stable enough to sustain the ESI circuit for a sheathless interface is obtained. As the charge of the inner surface is changed, so is the direction of the EOF and thus the polarity of the applied potential must be reversed. Various covalently bonded cationic monomer coatings such as 3-aminopropyltriethoxysilane (APS) [109, 110], [3-(methacryloylamino)propyl]trimethylammonium chloride (MAPTAC) [111, 112] and [(acryloylamino)propyl]trimethylammonium chloride (BCQ) [111] are frequently used in CE-ESI-MS. These monomers provide highly stable surfaces by covalent silanization of the capillary wall. Rapid CE separations using APS coated capillaries were performed in Paper I and II. The MAPTAC surface yields a slightly lower EOF than APS [112] and consequently this coating was favorably used in combination with FTICRMS for the analysis of complex samples, as presented in Paper VI. Although these monomer coatings have shown good utility for CE-MS, they still have limitations and drawbacks. The silanization of a surface involves rather elaborate column preparation procedures. Furthermore, monomers do not yield complete coverage of the silanol groups and the formed Si-O-C bonds are unstable at alkaline pH [110]. This restricts their use to acidic conditions as the net surface charge is usually reversed at pH of 5-6 [110, 113]. In Paper IV, the performance of three positively charged alkyllaminosilyl monomers, N-Trimethoxysilylpropyl-N,N,N-trimethylammonium chloride (TAC), Tetradecyldimethyl(3-trimethoxysilylpropyl)ammonium chloride (TeDAC) and Octadecyldimethyl(3-trimethoxysilylpropyl)ammonium chloride (ODAC) was evaluated for use in CE-ESI-MS. All three monomers yielded stable, non-bleeding surfaces with a reversed EOF high enough to sustain the sheathless ESI circuit. Furthermore, rapid peptide separations with high efficiencies could be performed with all monomer surfaces. The coated capillaries showed a linear pH dependence and the isoelectric points (pI) were found to be 8.7, 8.6 and 8.8 for the TAC, TeDAC and ODAC surfaces respectively. The investigated monomers differ regarding the length of alkyl chain and the chromatographic effects increased with increasing chain length, as would be expected. It was concluded (see Figure 10) that the TAC coated capillaries acted solely as a CE column, the TeDAC showed CEC tendencies and the ODAC modified capillaries functioned as OT-CEC columns, when using capillaries with an inner diameter of 25 µm.
Figure 10. A CEC evaluation of the monomer modified CE capillaries presented in Paper IV. The sample consisted of thiourea (1), naphthalene (2) and phenanthrene (3).

An attractive alternative to monomers is the use of polymer modified surfaces. In general, polymers yield a higher degree of surface coverage, which is beneficial. Physically adsorbed cationic polymers are becoming more interesting as they are easy to prepare with simple and fast capillary coating procedures. A wide diversity of physically adsorbed cationic polymers has been employed in CE-UV [107]. Out of these, only Polybrene [114] and variants thereof [111, 115] have extensively been used for CE-MS applications. In Paper V, a physically adsorbed in-house synthesized polyamine coating, PolyE-323 [116], was evaluated for the use in CE-ESI-MS. The PolyE-323 surface yielded a high anodal EOF in the pH range of 2-10, facilitating rapid CE-ESI-MS separations of peptides and proteins. The actual coating procedure takes less than one hour and the surface can easily be regenerated. Furthermore, the surface coating showed good reproducibility, good stability at low and high pH and is tolerant to organic modifiers and samples with a complex matrix. Throughout the experiments, no bleeding effects of the surface could be observed.

Both the alkylaminosilyl monomers evaluated in Paper IV and the PolyE-323 polymer surface presented in Paper V facilitates rapid CE-ESI-MS separations of peptides and more complex samples, such as protein digests (Figure 11). Furthermore, they generated a stable EOF high enough to be
compatible with a sheathless ESI interface. The monomers yield a lower surface coverage than the polymer. Yet, they can easily penetrate a porous structure, as for instance a monolithic sol-gel, to give specific characteristics.

Figure 11. CE-ESI-TOFMS separation of tryptically digested BSA using: (A) the polyE-323 polymer and (B) the ODAC monomer coated surfaces described in Paper V and IV respectively.
6 Analyzing biological samples

Although interesting results can be obtained when method developments are performed for peptide and protein standards, the true challenge for CE-MS lies in the analysis of complex samples, especially of biological origin. Proteomic and peptidomic research is important in order to understand the key functions in a cell of an organism. Furthermore, proteins and peptides are potential markers for disorder and disease in an organism, hence developing efficient, sensitive and accurate analytical tools is of great importance for clinical diagnosis. However, proteomic analyses are troublesome, since biological samples show great diversity and complexity and are susceptible to degradation. Aside from containing peptides and proteins, the samples usually include cells, salts, carbohydrates, phospholipids etc., all complicating the proteome analysis. Furthermore, the concentration of peptides and proteins may vary over several magnitudes and concentrations of pM or less put demands on the detection.

State-of-the-art analysis in proteomics incorporates two dimensional gel electrophoresis combined with subsequent extraction, digestion and MS (or MS/MS) detection using MALDI or ESI and in some cases combined with LC-MS (MS/MS) [117, 118]. This widely used and powerful tool provides accurate data of molecular mass, amino acid sequences and sometimes posttranslational modifications. However, gel-based separation techniques are time consuming, have quantification difficulties and cannot be directly coupled to MS. Consequently, there is a need to find faster, more accurate and sensitive analytical tools for clinical screening and proteomic research. LC and CE are attractive alternatives to gel electrophoresis.

Human body fluids such as blood (plasma and serum), cerebrospinal fluid (CSF), urine and saliva are all of great clinical interest [119]. Blood analyses are the most common and routinely performed in many clinical laboratories. However, from a neuro chemical point of view, the most interesting fluid is probably CSF. CSF is a physiological fluid mainly produced by the choroid plexus in the ventricles of the cerebral hemispheres of the brain. CSF flows from the paired ventricles in the cerebrum to a series of connected, unpaired cavities at the core of the brain stem. CSF is in continuum with the extracellular fluid of the central nervous system. Thus, it reflects certain aspects of the brain’s biochemical environment. A consequence of neurological disorders is often an altered level of specific CSF proteins or the total CSF protein level. This change can be used as an indicator of a specific
neurological disease. A large and rapidly growing number of disorders have been investigated for correlation with CSF proteins [120], including forms of amyloidosis [121] and psychiatric disorders such as schizophrenia [122], Alzheimer’s disease [123] and dementia [124].

Various approaches using MS as detection have been employed for proteomic and peptidomic studies of human CSF. These approaches include continuous infusion ESI-MS [119], one- and two-dimensional gel electrophoresis in combination with MALDI-MS, ESI-MS or LC-ESI-MS [125-130] and LC coupled to ESI-MS and MALDI-MS [131-133]. Although the results may vary depending on the approach used, it is clear that implementing a separation technique prior to the MS detection vastly improves the outcome of the analysis.

In Paper VI, CE in combination with ESI-FTICRMS was used for a proteomic analysis of human CSF. In this study, a so-called bottom up approach was employed [134]. The bottom up approach begins with a global enzymatic cleavage, followed by sample preparation, separation, MS detection and finally database mining. The CSF sample from a healthy male donor was tryptically cleaved and the tryptic digest was desalted and concentrated using off-line solid phase extraction (ZipTipC18) followed by centrifugation under vacuum to dryness. The reason for a bottom up approach is that on-the-fly MS/MS is difficult to perform as the peak widths in the CE separation are on the order of seconds and the acquisition rate of the FTICRMS instrument is rather limited. A miniaturized SPE step was implemented to remove salts and other contaminants, which otherwise would affect both the CE separation and ESI-MS analysis. Centrifugation under vacuum concentrates the sample and yields a sample more suitable for storage. However, increasing the number of steps in the analysis, especially conducted off-line, enhances the risk of sample loss and contamination. Approximately 40 nL redissolved sample was injected and separated by a MAPTAC and Black Dust coated CE capillary coupled to a 9.4 T FTICRMS instrument. Figure 12 shows an electropherogram of the CSF tryptic digest in a high resolving two-dimensional view.
The entire CE-ESI-FTICRMS run generated more than 10 000 peaks, which in turn could be deconvoluted to 1500 unique masses. Protein and protein precursor identification in the CSF sample was based on searches in a selected database [119] of proteins known to be present in human body fluids, primarily plasma and CSF. Peptide mapping rendered in a positive match for 14 proteins on a 99% confidence level, and 29 proteins (unfortunate double match of α-2-HS-glycoprotein in Paper VI) on a 95% level with mass measurement errors less than 5 ppm. The statistical evaluation has been described elsewhere [119]. Briefly, it is based on the product of likelihood ratios for the measured mass measurement error compared to random matches and the observed nonrandom distribution (connectivity) of the peptides in the peptide match.

The results of the proteomic analysis of human CSF using CE-ESI-FTICRMS show great potential. In Table I, the results of continuous infusion ESI-FTICRMS [119], CE-ESI-FTICRMS and LC-ESI-FTICRMS [132] using almost an identical bottom-up approach on human CSF from different individuals are shown.
Table I. Comparison of the number of proteins identified in CSF using the continuous infusion, CE and LC approach. The proteins in boldface were > 99% significant, the others > 95% significant.

<table>
<thead>
<tr>
<th>Continuous infusion</th>
<th>Capillary electrophoresis</th>
<th>Liquid chromatography</th>
</tr>
</thead>
<tbody>
<tr>
<td>serum albumin</td>
<td>serum albumin</td>
<td>serum albumin</td>
</tr>
<tr>
<td>transferrin</td>
<td>transferrin</td>
<td>transferrin</td>
</tr>
<tr>
<td>immunoglobulin G</td>
<td>immunoglobulin G</td>
<td>immunoglobulin G</td>
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<tr>
<td>cystatin C</td>
<td>immunoglobulin M</td>
<td>immunoglobulin M</td>
</tr>
<tr>
<td>perlecan</td>
<td>apolipoprotein A-IV</td>
<td>apolipoprotein A-IV</td>
</tr>
<tr>
<td>α-1-antitrypsin</td>
<td>α-2-macroglobulin</td>
<td>α-2-macroglobulin</td>
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<tr>
<td>complement C4</td>
<td>vitamin D-binding protein</td>
<td>vitamin D-binding protein</td>
</tr>
<tr>
<td>prostaglandin D synthase</td>
<td>AMBP protein</td>
<td>complement C4</td>
</tr>
<tr>
<td>α-2-glycoprotein 1</td>
<td>plasma kallikrein</td>
<td>complement C9</td>
</tr>
<tr>
<td>haptoglobin</td>
<td>uromodulin</td>
<td>complement C 1S</td>
</tr>
<tr>
<td>fibulin 1 isoform D</td>
<td>transthyretin (prealbumin)</td>
<td>complement C D</td>
</tr>
<tr>
<td>actin γ</td>
<td>hemopexin</td>
<td>α-2-HS glycoprotein</td>
</tr>
<tr>
<td>α-1-antichymotrypsin</td>
<td>fibrin 1 isoform A</td>
<td>apolipoprotein A-II</td>
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<td>α-1B-glycoprotein</td>
<td>prostaglandin D synthase</td>
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<td>coagulation factor IX</td>
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<td>α-1B-glycoprotein</td>
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<td>plasma kallikrein</td>
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<td></td>
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<td>s-100 calcium-binding protein A</td>
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As can be seen in Table I, the continuous infusion analysis rendered in a positive match for 6 proteins on a 99% confidence level, and 13 proteins on a 95% level. The LC analysis yielded 23 positive matches on a 99% level and 39 on a 95% level. The number of identified proteins using either CE or LC was more than twice the number for continuous infusion. Furthermore, CE or LC also identified roughly 50% or more of the proteins identified by continuous infusion. This clearly demonstrates the benefits of using a separation technique prior to MS analysis, as ion suppression, spectral overlapping and space charge effects are reduced. When comparing CE with LC, approximately 65% (19 out of 29) of the proteins identified by CE were also found in the LC analysis. The divergence in obtained results for the three techniques can be explained by fluctuations in the ESI source over time, instrumental drift affecting the mass accuracy, non specific losses in the sample preparation and that the CSF samples originated from different healthy donors. Furthermore, peptide mapping is probabilistic and not a definite identification. It is also interesting to compare the injection volumes and analysis times for the techniques. In the CE analysis, about 40 nL was injected, which was 55 times less than for continuous infusion (2.2 µL) and 250 times less than for the LC analysis (10 µL). The analysis time for the CE experiments was half the time for continuous infusion and approximately 1/6 of the time required for the LC experiments.

CE is however not as robust as LC, has limited sample loading capabilities and difficulties with quantitative analysis. As such, the results obtained for the CE-ESI-FTICRMS analysis of human CSF show the potential for rapid and low sample consuming pre-studies of biological fluids. Based on the CE results, a more comprehensive LC method can be developed. Finally, it should be mentioned that the results obtained using either CE or LC are well comparable to 2D-PAGE coupled to MS. Yet, one drawback with the applied bottom up approach compared to traditional gel electrophoresis methods is the lack of identification of isomer forms of a protein.
7 Future aspects

Capillary electrophoresis coupled to mass spectrometry is today a fairly established technique. However, there are still issues that must be further developed in order to bring CE-MS to be the method of choice for routine analyses. Looking closer at CE-ESI-MS, new approaches to improve sample load capability, electrospray stability and to avoid analyte-wall interactions are constantly reported. However, the major issue, especially for the analysis of complex biological samples, is how to perform repeatable and reproducible quantitative analysis. A lot of the presented proteomic CE-MS analyses (including Paper VI) show proof-of-principle and a great potential for qualitative determinations. Still, many neurological disorders and conditions are expressed in altered protein levels [126]. Thus, reliable CE-MS approaches for quantitative proteomic research must be developed to bring the technique into routine use.

Multidimensional hyphenation of CE and other separation techniques is continuously growing. This is especially important for the separation of complex biological samples where a one-dimensional approach often is insufficient. In 2-D gel electrophoresis, it is common to separate the analytes according to their isoelectric point (pI) in the first dimension by isoelectric focusing in a pH gradient. The separation in the second dimension is based on the molecular weight of the analytes. A similar approach can be utilized in capillary formats by combining capillary isoelectric focusing (CIEF) and CE [135]. Another interesting hyphenation is to combine LC and CE [75, 136]. Besides from yielding additional complementary information (hydrophobicity and size-to-charge ratio), LC-CE hyphenation also increases the sample loading capabilities. Although the combination of various separation techniques complicates the overall analysis, multidimensional hyphenation of CE and other orthogonal separation techniques are expected to increase in use [137].

Last, but maybe most important, is the strong trend to downscale electrodriven separation techniques into microfabricated devices or microchips. One goal with microchips is to implement all steps in the analysis; sample injection, sample preparation, separation and detection into a fully integrated and automated micrototal analytical system (µ-TAS). These systems are predicted to become the most powerful analytical tools for applications in life sciences [138], offering rapid, high efficient, high sensitive and high throughput analysis. These systems, implementing free
zone electrophoresis, can be combined on-line with ESI-MS. The Black Dust and Black Jack electrospray and nanoelectrospray emitters developed in Paper I-III and the monomer and polymer surface coatings described in Paper IV-V can be utilized in μ-TAS systems. This is currently pursued in our lab with the goal to eventually perform analysis with disposable chip formats on such complex biological samples as described in Paper VI.
8 Concluding remarks

This thesis describes the current status and aspects of capillary electrophoresis coupled to mass spectrometry using (sheathless) electrospray ionization. During the past 15 years since CE-ESI-MS first was introduced, a lot of initial bottlenecks have been overcome. One major limitation in early reports of sheathless CE-ESI-MS was the limited lifetime of the electrospray emitter. Today, several approaches, including the Black Dust and Black Jack methods presented in Paper I and II, have shown improved long-term stability of the emitters. The failure of the emitter is often caused by electrochemical reactions occurring at the emitter-liquid interface, as a consequence of the controlled current electrochemical cell behavior of electrospray. The electrochemical properties of the emitter can be evaluated by classical electrochemical methods, such as cyclic voltammetry and chronoamperometry. This was demonstrated in Paper III, where metal, polymer and graphite coated emitters were investigated. The graphite coated emitters showed good electrochemical stability compared to the other emitter configurations, which also has been demonstrated in electrospray and nanoelectrospray mode.

Analyte-wall interactions have long been known to be a problem in CE analysis. This can be circumvented by internally modifying the capillary walls. Furthermore, it is of outermost importance to have a stable and sufficiently high EOF to sustain the electrospray, when using a sheathless approach. In Paper IV and V, new monomer and polymer coatings were presented for rapid and high-efficient separations of peptides and proteins/protein digests.

In Paper VI, the use of CE-ESI-FTICRMS showed great potential for rapid proteomic probing of human cerebrospinal fluid. The results were comparable with more established techniques, such as LC-MS and 2-D gel electrophoresis coupled to MS, with lower sample consumption and faster analysis time. However, these techniques should not be regarded as competitive, rather complementary. The applications and use of CE-ESI-MS will continue to grow as the current trends of multidimensional hyphenation and microfabricated devices are further developed and explored.
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Proteiner och peptider har en betydande roll för cellernas funktion i en organism. Varje cell innehåller tiotusentals olika proteiner i varierande koncentration. Det är av yttersta vikt att kunna mäta proteininnehållet i en cell, ett organ eller en kroppsvätska, dels för att fastställa deras funktion i en organism men också för att klargöra huruvida proteiner (och peptider) är involverade i olika sjukdomsförlopp.

Ända sedan tidigt 30-tal har elektrofores varit en av de dominerande separationsmetoderna för proteinanalys. Separationsmekanismen i elektrofores bygger på att joner i en lösning eller gel, beroende på storlek och laddning, vandrar med olika hastighet mot respektive motpol när ett högspänningsfält läggs över lösningen eller gelen. Kapillärelektrofores (CE) är en utveckling av elektrofores. I CE sker separationen i silikakapillärer med inner- och ytterdiameter i mikrometerstorlek. Fördelen med denna nedskalning är högupplösende och effektiva separationer av t.ex. peptider och proteiner.

Masspektrometri (MS) är idag en av de mest sofistikerade och kraftfulla mättekniker som finns tillgänglig inom analytisk kemi, med ett brett tillämpningsområde. En masspektrometer separarerar och mäter joner enligt deras massa till laddningsförhållande. Med hjälp av en masspektrometer kan analyternas massa och struktur bestämmas med stor noggrannhet. Vidare är MS en känslig mätteknik som möjliggör detektion av substanser i låg halt.

Olika vätskebaserade separationstekniker, såsom kapillärelektrofores och vätskechromatografi, kan med fördel kombineras med masspektrometri. Ett av de största problemen i denna kombination är att separationen av analyterna utförs i vätskefas, medans detektionen i masspektrometern sker i vakuum. En stor utveckling har skett inom själva kopplingen mellan vätskebaserade separationstekniker och masspektrometri, d.v.s en utveckling av metod för att överföra joner från vätskefas till gasfas, även kallade joniseringstekniker. Elektrospray är idag en av de mest lyckade och tillämpade joniseringsteknikerna. Grunden för elektrospray är snarlik elektrofores och kan även liknas vid en elektrokemisk cell. I elektrosprayjonisering läggs ett högspänningsfält över kopplingen mellan vätskefaseparation och masspektrometer. Detta högspänningsfält kommer att ge upphov till en ström av joner, med riktning mot och in i masspektrometern. Strömmen upprätthålls av elektrokemiska reaktioner, vilka i sin tur leder till att nya joner kontinuerligt skapas och lämnar
vätskefasen. Tack vare utvecklingen av bl.a. elektrosprayjonisering har masspektrometri erhållit ett enormt genomslag i biokemisk, biologisk och klinisk forskning. Orsaken till detta är att intakte proteiner och proteinkomplex via elektrosprayjonisering kan undersökas med masspektrometri.


Slutligen diskuteras gällande trender och framtida utvecklingar för CE-ESI-MS. Kapillärelektrofores har, och kommer i allt större grad, kombineras med andra vätskebaserade separationstekniker, såsom miniaturiserad
vätskekromatografi, för masspektrometrisk analys av komplexa biologiska prover. Den starkaste trenden är dock utvecklingen av analytiska plattformar i miniatyriserade chipformat. I dessa format kommer hela analyskedjan bestående av provtagning, provbehandling, separation och detektion integreras och automatiseras. Kapillärelektrofores och masspektrometri kommer med största sannolikhet ha stor betydelse i dessa chipformat.

*That's all folks!*
11 References


A doctoral dissertation from the Faculty of Science and Technology, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Comprehensive Summaries of Uppsala Dissertations from the Faculty of Science and Technology. (Prior to October, 1993, the series was published under the title “Comprehensive Summaries of Uppsala Dissertations from the Faculty of Science”.)