Interfacing Complementary Separation Techniques with Mass Spectrometry Utilizing Electrophoresis, Nanoparticles, and Functionalized Magnetic Beads

DAVID MALMSTRÖM
Capillary electrophoresis (CE) has during the last two decades become more robust and been able to separate neutral analytes without compromising the downstream detection. An interesting aspect in CE compared to more commonly used high-performance liquid chromatography is the orthogonal separation mechanism provided by CE. Compounds are separated based on charge and size with extremely high separation efficiencies. However, since mass spectrometry (MS) has become one of the most important analytical detectors and play a key role for pharmaceutical- and in clinical applications it is of major importance that the two techniques successfully can be combined without any compromises. Improvements in existing ion sources must be made in order to fully take advantage of the potential in capillary electrophoresis and mass spectrometry. One way is to miniaturize the ion source (paper I) in order to make it more compatible with the smaller liquid volumes and lower flow rates in CE. Despite these improvements challenges such as low sample concentrations, non-separated peaks, unspecific losses, and poor ionization still remain, and are addressed in this doctoral thesis.

Separation of neutral analytes has previously been achieved with packed columns but with several disadvantages. Therefore, MS-compatible pseudostationary phases in the form of nanoparticles (paper II) are an interesting alternative with its minimized risk of clogging, reduced memory effects and better separation efficiencies. Particles or beads have also shown to be of importance when reducing the dynamic range in complex samples. By creating functionalized magnetic beads (paper III), complex samples such as human plasma can be fractionated in the manner that low molecular weight proteins are selectively enriched. Despite fractionation and enrichement of analytes of interest (paper IV) the ionization suppression could lead to biased sensitivity, increased baseline, retention variations and chromatographic distortion. Therefore the separation, as well as the ionization, is of major importance. For instance, in order to separate and detect monoclonal antibodies, which are an upcoming class of biotherapeutic drugs, the choice of capillary temperature and sheath liquid composition must be considered due to its major influence on charge state, peak intensity and memory effects (paper V).

David Malmström, Uppsala University, Department of Physical and Analytical Chemistry, Analytical Chemistry, Box 599, SE-751 24 Uppsala, Sweden.

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ISSN 1651-6214
ISBN 978-91-554-8529-0
urn:nbn:se:uu:diva-183666 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-183666)
“The scientist is not a person who gives the right answers, 
He is one who asks the right questions.”

Claude Lévi-Strauss
List of Papers

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


The author’s contribution to the papers

Paper I:
Took part in planning the study, performed the experiments together with JA and took part in writing the paper.

Paper II:
Planed the study together with PS, performed the experiments and wrote the paper.

Paper III:
Planed the study together with MW, performed the experiments and wrote the paper.

Paper IV:
Took part in planning the experiments, performed the MALDI analysis and aided in writing the paper.

Paper V:
Planed the study together with MP, performed the experiment and wrote the paper.

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Papers not included in this thesis

i Shevchenko, G., Sjödin, M. O. D.†, Malmström, D.†, Wetterhall, M., Bergquist, J. (2010) Cloud-Point Extraction and Delipidation of Porcine Brain Proteins in Combination with Bottom-Up Mass Spectrometry Approaches for Proteome Analysis. *Journal of Proteome Research, 9*(8) 3903-3911


† Authors contributed equally to the manuscript
Contents

1. Introduction ................................................................................................................. 11

2. Capillary Electrophoresis ...................................................................................... 14
   2.1. General concept of Capillary Electrophoresis .............................................. 15
   2.2. Electroosmotic Flow ................................................................................. 16
   2.3. Capillary Modifications ........................................................................... 17

3. Electrokinetic Chromatography ........................................................................... 18
   3.1. Why Pseudostationary Phases? .............................................................. 18
   3.2. Micellar Electrokinetic Chromatography .............................................. 19

4. Mass Spectrometry .............................................................................................. 21
   4.1. The use of Mass Spectrometry ............................................................... 22
   4.2. Ion Sources ............................................................................................ 22

5. Modifications in Capillary Electrophoresis-Mass Spectrometry ...................... 25
   5.1. Tapered Capillaries ............................................................................... 26
   5.2. Requirements of the Interface ................................................................ 26

6. Adaptation of Pseudostationary Phases to Mass Spectrometry ......................... 32
   6.1. Micellar Electrokinetic Chromatography- Mass Spectrometry .............. 33
   6.2. Continuous Full Filling .......................................................................... 34

7. Particles, Beads, and Other Separation Media .................................................... 38
   7.1. New PSP’s with Improved Mass Spectrometric Compatibility .......... 39
   7.2. Magnetic Beads ..................................................................................... 40
   7.3. Functionalized Magnetic Beads ............................................................. 42
   7.4. Antibodies and Immunoaffinities ......................................................... 44

8. Concluding Remarks ............................................................................................... 46

9. Swedish Summary ................................................................................................. 48

10. Acknowledgments ................................................................................................. 51

11. References ............................................................................................................. 53
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>APPI</td>
<td>Atmospheric Pressure Photoionization</td>
</tr>
<tr>
<td>APCI</td>
<td>Atmospheric Pressure Chemical Ionization</td>
</tr>
<tr>
<td>BGE</td>
<td>Background Electrolyte</td>
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<td>CFF</td>
<td>Continuous Full Filling</td>
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<tr>
<td>CE</td>
<td>Capillary Electrophoresis</td>
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<td>CEC</td>
<td>Capillary Electrochromatography</td>
</tr>
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<td>CMC</td>
<td>Critical Micellar Concentration</td>
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<tr>
<td>CZE</td>
<td>Capillary Zone Electrophoresis</td>
</tr>
<tr>
<td>EKC</td>
<td>Electrokinetic Chromatography</td>
</tr>
<tr>
<td>EOF</td>
<td>Electroosmotic Flow</td>
</tr>
<tr>
<td>ESI</td>
<td>Electrospray Ionization</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
</tr>
<tr>
<td>i.d.</td>
<td>Inner Diameter</td>
</tr>
<tr>
<td>iMALDI</td>
<td>Immunoaffinity MALDI</td>
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<td>ITP</td>
<td>Isotachophoresis</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal Antibody</td>
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<tr>
<td>MALDI</td>
<td>Matrix Assisted Laser Desorption/Ionization</td>
</tr>
<tr>
<td>MEKC</td>
<td>Micellar Electrokinetic Chromatography</td>
</tr>
<tr>
<td>MS</td>
<td>Mass Spectrometry</td>
</tr>
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<td>MS/MS</td>
<td>Tandem Mass Spectrometry</td>
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<tr>
<td>o.d.</td>
<td>Outer Diameter</td>
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<td>PF</td>
<td>Partial Filling</td>
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<td>PSP</td>
<td>Pseudostationary Phase</td>
</tr>
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<td>RM</td>
<td>Reverse Migrating</td>
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<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulfate</td>
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1 Introduction

Electrophoresis, which is the separation of charged molecules based on different migration in an applied electric field, has since it was introduced by Tiselius [1] in the 1930’s and later on adapted into capillary formats by Hjer- tén and others [2-4] developed into a robust separation technique. John Craig Venter et al. [5] showed in 2001 the great power of capillary electrophoresis (CE) when the human genome was sequenced well ahead of schedule. However, there are some limitations with CE, such as the inability to separate nonionic solutes and separate analytes with the same size-to-charge ratio. There have been ways to solve these limitations, such as packed capillaries, capillary electrochromatography (CEC), and the use of pseudostationary phases (PSP). The basic principal of CEC is that a fused silica capillary is packed with a stationary phase. The packing materials are typically the same as for conventional high pressure liquid chromatography (HPLC), such as bonded silica and ion exchangers or monoliths. The most obvious advantage of CEC is that it combines the features of CE, with its flat-flow velocity profile generated by the electroosmotic flow (EOF) and reduced peak broadening, with the selectivity in HPLC. However, ever since the introduction of CEC it faced some major challenges. For instance, the method of packing the capillaries was problematic, since bubble formations and frit problems arises and could lead to column-to-column variation. This will affect the EOF that is controlled mainly by the surface of the packing material. Due to the difficulties in the packing of the capillaries and thereby varying electroosmotic flow, traditional CEC can be seen as irreproducible in a larger perspective. There have been many different ways of improving the packing of solid particles into the capillaries; capillaries with pressurized ends [6], sol-gel methods with in-situ polymerization [7] and gel based CEC [8]. But, the challenges with producing good frits still exist. [9] Despite the challenges, development in CEC is still active since the breakthrough of CEC in the second half of the 1990’s.

Another way to circumvent the limitations of packed columns is to use a pseudostationary phase. This interesting technique was demonstrated by Terabe and others in 1984 where a solution of ionic micelles was introduced in the background electrolyte [10-14]. The separation principle was however first mentioned by Nakagawa [15] and is based on the migration of micelles in an aqueous solution by electrophoresis - micellar electrokinetic chromatography (MEKC).
The use of mass spectrometry (MS) has during the past 25 years reached an outstanding position among analytical methods due to its sensitivity, specificity and low detection limits. One of the areas where MS has become the method of choice is in the enormous field of complex protein samples – proteomics [16]. Mass spectrometry has also been recognized by the Nobel Committee by awarding the Nobel Prize in chemistry in 2002 for two ionization techniques; electrospray ionization (ESI) and laser desorption ionization (LDI). Olivares et al. [17] and Smith et al. [18] were the first to combine the powerful technique of mass spectrometry with capillary electrophoresis (Figure 1) by developing a new interface. The CE compatible interface was based on ESI initially developed by Dole et al. [19, 20] and Fenn et al. [21, 22]. Due to their great work CE-MS is now routinely used in areas such as metabolome analysis [23] and purity analysis in the drug industry [24].

*Figure 1.* This thesis covers how to interface complementary separation techniques with mass spectrometry utilizing capillary electrophoresis, nanoparticles, and functionalized magnetic beads.

During the last decade, nanoparticles have been a hot topic. The use of nanoparticles as pseudostationary phases in combination with continuous full filling (CFF) and an orthogonal ESI-interface has shown to be an effective way of separating neutral analytes in CE without affecting the electrospray process or contaminating the ion source ([paper II] [25, 26]). An orthogonal method of coupling MEKC and MS utilize an alternative ionization source – atmospheric pressure chemical ionization (APCI) and atmospheric pressure photoionization (APPI) [27]. In these methods the ionization efficiency has been shown to be unaffected by the surfactants using both APCI and APPI [27-29].

Nanoparticles with magnetic properties or magnetic beads are an area that has gained a large interest during the last decade. The unique properties have led to their wide use as a solid support in a variety of applications, such as
drug deliverers, support for immobilized enzymes, bioaffinity adsorbents, facilitating magnetic resonance imaging for medical diagnosis and helping fractionating complex samples.

This doctoral thesis is based on fundamental studies showing how the separation as well as the coupling of CE with MS can be improved. In paper I, the detection sensitivity in APPI-MS was optimized by varying different ion source parameters. The results indicated that a specially designed APPI interface for low flow rates is favorable. In paper II, a pseudostationary phase involving nanoparticles and the continuous full filling technique was investigated. Both separation and ion source parameters were evaluated and optimized leading to improvements in column-to-column variability and detection limits. Yet, another type of bead was investigated in paper III, a functionalized magnetic bead. The basic principle with this type of bead is the ability to fractionating high molecular weight proteins while simultaneously enriching low molecular weight proteins. This is especially useful in the field of proteomics, when for instance searching for biomarkers for treatment of diseases. Another method for enrichment and separation was presented in paper IV, where a thin layer of mesoporous titanium dioxide was sintered onto a conductive glass slide for analysis of multi- and mono-phosphorylated peptides with Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry.

Monoclonal antibodies have shown good progress in the development for many different disease indicators. However, the separation and detection of monoclonal antibodies has mostly been performed with non-universal detectors or including tedious method developments. In paper V, the first study of intact monoclonal antibodies using non-coated capillary electrophoresis coupled to electrospray mass spectrometry was presented. Consistent with the other included papers in this thesis fundamental parameters were investigated. For instance, the effect of sheath liquid, mass spectrometry parameters and capillary temperature were studied in order to achieve an optimal ionization, system stability and thereby optimized detection and identification.
2 Capillary Electrophoresis

"The science of today is the technology of tomorrow"

Edward Teller

Capillary electrophoresis is a collective term for many different methods. The general benefit of CE is the highly efficient separations for all the electrophoretic driven separations. However, the inability to separate neutral analytes with traditional capillary zone electrophoresis (CZE) is a major limitation. Capillary electrochromatography (CEC) was a way of solving this challenge where a capillary was packed with e.g. the same stationary phase as in high pressure liquid chromatography. Although CEC and HPCL are very similar in many ways, one main difference is the flat flow velocity profile generated by the electroosmotic flow in CEC, in comparison to the pressure driven parabolic flow velocity profile in HPLC. This difference in front profile lead to the highest separation efficiencies of all time. Unfortunately, the reproducibility was a major challenge in CEC. The problem turned out to dependent on the heterogeneous surface charges resulting in local variations in EOF. Despite several approaches to minimize the problems by improving the packing methods, improved frit fabrications and in-situ polymerization the reproducibility still remained a challenge [9].

The predecessor of capillary electrophoresis, gel electrophoresis, utilized an efficient way of avoiding joule heating, but unfortunately led to convection and band broadening. When trying to exclude the gel for a liquid based system, it turned out to be impossible to separate neutral analytes and analytes with the same size-to-charge ratio. Gel filled capillaries, similar to traditional gel electrophoresis, has also been investigated. However, producing and using gel filled capillaries is often quite difficult due to bubbles formed inside the gel. The challenge was elegantly solved by using non-cross-linked gels since the gel could be injected into the capillary and after separation discarded [30, 31]. Apart from the non-cross linked acrylamide gels, other types of non-trapped stationary phase have been used in CE or CEC and will be further discussed in chapter 3.
2.1 General concept of Capillary Electrophoresis

The separation by electrophoresis is based on different migration of charged compounds (ions) by attraction or repulsion in an electric field\cite{2,3}. In CE, the electrophoretic separation is taking place in a thin glass capillary made of fused silica. The inner diameter (i.d.) of the capillary is typically 25-75 µm, which can be compared to a human hair strand, typically at 80 µm in diameter. The outer surface of the capillary is coated with a polymeric substance, polyimide, which gives a tremendous flexibility to the otherwise very fragile capillary. In comparison to traditional gel electrophoresis, CE is filled with a buffer. The choice of buffer has a major influence since it will provide contact closure to the applied high electric field. In gels this applied current is in higher degree limited by Joule heating, the heat of a conducting medium as current flows through it. This will have an effect on the speed of the separation due to the limitation in using a higher current and potential. Electric fields between 100 and 500 V/cm are possible with only minimal heat generation. With higher electric fields come shorter analysis times, higher peak efficiencies and better resolution. The inner diameter of the capillary also has a large effect on the limitations. An increase in i.d. leads to a lowering of the surface-to-volume ratio. A capillary with 50 µm in i.d. will have around 78 times higher surface-to-volume ratio compared to a standard slab gel and 1.5 times higher than the capillary with 75 µm in i.d. The high surface-to-volume ratio will allow very efficient dissipation of Joule heat generated from the high applied electric fields. As a result, electrophoretic separations in capillary electrophoresis can easily be performed at up to 30 000 volts. Though, studies using up to 60 000 V in non-aqueous solutions has been demonstrated\cite{32}.

The simplest form but also the most commonly utilized type of CE is capillary zone electrophoresis (CZE) (see Figure 2). The capillary is filled with an appropriate separation buffer with a desired pH, where the analytes get charged. The sample is injected in the inlet, normally the anode end while the outlet is grounded either by placing in another buffer vial or adding an extra liquid flow called sheath liquid. As a high voltage is applied into the two buffer reservoirs, ionic species in the sample plug will start to migrate with an electrophoretic mobility determined by their charge and mass. However, if the applied electric field where the only driving force acting on the ions only net positively charged compounds (cations) would be separated, neutral compounds would remain static, and net negatively charged compounds (anions) would be driven back into the buffer vial (inlet end). CZE would therefore be quite limited in its use. Fortunately, a force called electroosmotic flow (EOF) creates a movement of all components in the capillary towards the cathode (unless a modified capillary is used, see chapter 2.3).
Figure 2. In Capillary Zone Electrophoresis, negatively charged silica surface in combination with the applied electric current creates an electroosmotic flow (EOF). Neutral components (N) will migrate along with the EOF while charged analytes (- and +) will be migrating with an electrophoretic mobility determined by their charge and mass.

A marker is useful to add as an internal standard in order to determine relative migration times for the separated compounds. In CZE, a neutral compound, e.g. dimethyl sulfoxide (DMSO) is used since only the charged species can be separated and the neutral analytes will migrate with the EOF. Herein lay also a major problem with CZE. The limiting factor of not being able to separate neutral components has been solved by the development of Electrokinetic Chromatography (EKC). Within EKC, pseudostationary phases such as micelles and nanoparticles (see chapter 3) has emerged as important additives.

2.2 Electroosmotic Flow

Silanol groups (SiO\(^{-}\)) on the inside wall of the capillary will attract cationic species from the introduced buffer. This attracted ionic layer has a positive charge that will decrease exponentially with the distance from the capillary wall increases. The double layer that is formed closest to the surface is named Stern Layer or Inner Helmholtz \(^{[33]}\) and can be seen as a static layer.
A mobile and more diffused layer is formed distal to the Stern Layer, called Outer Helmholtz Plane (OHP). When a field is applied, cations will start to move in the OHP towards the cathode and carrying water molecules at the same time. The cohesive nature of the hydrogen bonding of the waters of hydration to the water molecules of the bulk solution will result in the entire buffer solution being pulled towards the cathode. This bulk flow is called electroosmotic flow (EOF) and can be seen as a driving mechanism that will carry all analytes. A flat flow front profile will be created by the EOF. The flat flow profile will affect all analytes in the same way leading to sharp peaks, in opposite to the parabolic flow present in HPLC (see Figure 2). The background electrolyte (BGE) will have a large effect on the EOF due to the pH dependency of the silanol groups at the capillary inner wall. Apart from pH, the ionic strength will have a large effect on the EOF. High ionic strength or electrolyte concentration will compress the Stern Layer, decrease the zeta potential (the potential between two layers of the opposite charge) and thereby reduce the mobility of EOF. Optimizing the EOF can therefore permit shorter analysis time while still having well separated peaks.

2.3 Capillary Modifications

Basic proteins and peptides have a high tendency to interact with the capillary wall resulting in low efficiencies, low recovery, decreased sensitivity, and poor reproducibility. Extreme pH values, high salt concentrations and various BGE additives have been ways of modifying the capillary in order to circumvent adsorption and to improve the separation. However, when using ESI/MS the preferable strategy has been to permanently coat the inner capillary wall. The permanently coatings are attached prior to analysis and are therefore suitable with ESI/MS by excluding it from the buffer electrolyte. Despite the regeneration of the coatings by flushing the capillary in between injections, the time, stability, repeatability issues and cost required to prepare the coating is a challenge to overcome.

Reducing, neutralizing or reversing the EOF can be made with capillary coatings. Gilges et al. showed how polyvinyl alcohol and other polymers can be used as a dynamic coating. The coating effectively suppressed the EOF resulting in more focused zones. This method later developed into isotachophoresis (ITP) and isoelectric focusing (IEF). Except modifications on the inner capillary wall, coatings on the tip and outside wall with metalized coatings and emitters has been developed in order to secure the electrical connection between the capillary end and the ESI source. This type of modification has shown an important role in sheathless ESI, and will be further discussed in chapter 5.2.
3 Electrokinetic Chromatography

“Never, never, never give up”

Winston Churchill

Capillary zone electrophoresis cannot separate neutral analytes due to the lack of electrophoretic mobility, and hence came the development of electrokinetic chromatography (EKC). In 1982, Terabe and co-workers \cite{49} published the first successful separation with sodium dodecyl sulfate (SDS) as an additive in the buffer. The technique was later termed micellar electrokinetic chromatography (MEKC). Apart from micelles, other types of additives have been developed and used in EKC. How the additives are introduced into the flow has been varied to be compatible with mass spectrometry without ion suppression and ion source contamination.

3.1 Why Pseudostationary Phases?

The most frequently used separation technique for analysis in liquids is HPLC. However, since conventional setup requires large sample and buffer volumes, miniaturized microLC and nanoLC systems \cite{50,51} have gained in popularity. To improve the separation efficiency, the particle size for the stationary phase has been decreased in parallel to the instrumentation miniaturization \cite{52}. Despite any improvement with smaller particle sizes, the nature of a packed stationary phase permit a risk of sample adsorption and column clogging and would get higher with decreased column size. The challenge with memory effects is another problem with stationary phases e.g. HPLC and CEC. It is therefore not a coincidence why the use of “moving stationary phases” i.e. pseudostationary phases, as in paper II, has gained in popularity \cite{49,53}.
3.2 Micellar Electrokinetic Chromatography

Terabe was one of the pioneers in introducing selectivity in CE by the use of electrokinetic chromatography [10-14]. However, it was later termed micellar electrokinetic chromatography (MEKC) to specify the use of micelles. These initial reports resulted in an explosion of studies during the 1990’s with the use of different surfactants, polymers, macromolecules and particles as pseudostationary phases (PSP’s) [14, 49, 54, 55]. The technique was based on partitioning analytes between two phases, a pseudostationary phase composed of charged micelles and the surrounding electrolyte phase migrating at different velocities. Terabe generated anionic micelles out of SDS and showed that the micelles had an electrophoretic mobility directed towards the positively charged anode. Despite the electrophoretic mobility directed towards the capillary inlet, micelles will migrate towards the detector due to the high EOF. Analytes would be partitioned between the micellar phase and the aqueous phase. A neutral analyte, which will spend no time with the micelles, will travel with a migration time of $t_0$, while the neutral analytes that will spend all of its time with the micelles will migrate with the migration time $t_{mc}$. Other neutral analytes will partition themselves in between the two phases and thereby migrate at intermediate times (see Figure 3).

![Figure 3](image)

*Figure 3.* Schematic figure of the separation mechanism in MEKC using anionic micelles. The principle is similar to the traditional liquid chromatography, i.e. the sample is distributed between two phases migrating with different speeds. But in contrast to traditional chromatography, the pseudostationary phase in EKC has an electrophoretic mobility. Despite the fact that the micelles are strongly negatively charged and therefore should migrate towards the injector end, the net mobility will be towards the detector side due to the high EOF under neutral and alkaline conditions.

Adding SDS to the background electrolyte permits separation of anionic, cationic, and neutral analytes in the same run. The separation of neutral analytes will be based on the differences in hydrophobicity and hydrogen bonding, and can be controlled by varying surfactant concentration, pH and the organic additives e.g. methanol or acetonitrile. The surfactant or micelle in MEKC can be compared to the stationary phase in conventional liquid...
chromatography. The selectivity in MEKC depends on the structure of the surfactant molecule. The selectivity can therefore be manipulated by varying the polar head group \[^{[56]}\] or mixing different surfactants. However, the selectivity can be varied by modifying the surfactant concentration, the choice of buffer, pH of electrolyte solution or the temperature. But the most convenient way is to incorporate or modify an organic solvent to the BGE. This was shown by S.K. Poole and C.F. Poole \[^{[56, 57]}\] to be the most efficient way to manipulate the selectivity. The buffer composition, concentration, pH, temperature, and voltage turned out to only have a small influence \[^{[58]}\].
4 Mass Spectrometry

“The farther an experiment is from theory, the closer it is to the Nobel Price”

Irène Joliot-Curie

Since the 1980’s mass spectrometry has reached an unsurpassed status as analytical tool because of its universality, sensitivity, selectivity and ability to give the elemental composition, structural information and qualitative and quantitative information of analytes [59-63]. Although several different types of MS instrumentations exist, they all include three main parts. The first part is an ionization source where the analytes are ionized and transferred into the gas phase, a mass analyzer that will sort the analyte ions by their mass-to-charge (m/z) ratio, and finally a detector which will monitor the separated ions. Tandem mass spectrometry (MS/MS) involves an extra step. An extra mass analysis will occur after the ions with a specific m/z are fragmented. Fragmentation can occur through addition of energy or by collision with a gas. The benefit of MS/MS is the valuable information obtained regarding molecular structures of an unknown analyte due to the often unique fragmentation pattern for a specific analyte. Other benefits of MS/MS are the possibilities of higher selectivity and sensitivity. [64, 65]
4.1 The use of Mass Spectrometry

Mass spectrometry has gained an enormous impact in many important fields such as proteomics, peptidomics, and metabolomics \[66, 67\]. This is due to the introduction of the soft ionization techniques, electrospray ionization (ESI) and Matrix Assisted Laser Desorption/Ionization (MALDI). These techniques made it possible to analyze intact biological molecules, such as proteins, peptides, and polymers. The use of mass spectrometry has become an important part in the large scale study on the proteome, proteomics. Except the use of MS to discovering potential biomarkers for severe diseases like Alzheimer’s and Parkinson’s disease \[68\], MS has been used in other fields such as forensic science. The use of gas chromatography coupled to mass spectrometry have shown to be an important tool when detecting drug abuse in athletes, or detect accelerants in arson investigations. Modified MS instrumentations, so-called space MS is valuable in NASA’s Origins theme and have been traveling in space since the early 1960s and have returned information about the solar wind isotopes and other isotopic ratios. \[65\] High demands are put on the instrumentation since MS can be used in so many different fields of interest. The mass spectrometer need to be fast in order to keep up with hyphenated techniques and be sensitive enough to detect low amounts, but at the same time give reproducible results.

4.2 Ion Sources

In 1955 O’Konski and Doyle \[69\] started working on a new interesting technique that later Richardson \[70\] continued. Richardson sprayed a diluted polymer solution onto a carbon coated film. The solvent was allowed to spread out in the carbon surface and thereafter be evaporated. The remaining single solid entities on the carbon surface could then be photographed in an electron microscope. This turned out to be a forerunner to the most commonly used ionization technique today, electrospray ionization (ESI). In the late 1980’s ESI was combined with MS due to the work by Fenn and co-workers. In ESI a sample solution enters the ion source through a stainless steel needle. The needle is maintained at a few kilovolts relative to the surrounding chamber. As a result, the electric field at the needle tip will charge the introduced liquid. Due to Coulomb forces the liquid will disperse into a fine spray of charged droplets. The applied drying gas will evaporate the solvent, leading to smaller droplets but with higher charge density at the surface. This may then lead to a Coulomb explosion where the droplet tears apart. This progression of events is repeated until ions exist in ambient gas. The electric field will let the droplets and ions migrate towards the inlet of the MS.
One of the latest among the soft ionization techniques is the atmospheric pressure photoionization (APPI) source \cite{71}. This ionization technique has shown less dependency on buffer composition compared to the ESI source and is compatible with low flow rates. This indicates that APPI is suitable for CE due to the commonly used non-volatile buffers and low flow rates \cite{72}. In contrast to ESI, the spray needle is introduced into a heater where the sample is vaporized (Figure 4). The ionization process will then be initiated by a discharge lamp. One of the major benefits with APPI is the possibility to discriminate between the analyte and solvent ions. By selecting a suitable UV source, so that the selected photon-emission energy is higher than the ionization energies (IEs) of the target molecules, but lower than the IEs of the solvent and surrounding air it is possible to take full advantage of the APPI source. Therefore, the krypton lamp (10.0 eV) is probably the most suitable UV source since it has a photon energy lower than the major components of air and the most commonly used solvents. For instance, hexane, isopropanol, methanol, oxygen, acetonitrile, water, and nitrogen all have
higher IE than the energy of the emitted photons by the krypton lamp and are therefore not ionized. The probability to directly ionize an analyte molecule is not very likely to occur, and therefore a so-called dopant is introduced. This was shown by Sprangler et al. \cite{73} when the number of ions was significantly increased by introducing a suitable substance. A dopant is effective if it is photoionizable and can function as an intermediate between the photons and the analyte, like the typical dopant acetone (9.70 eV).

Matrix Assisted Laser Desorption/Ionization is, as ESI and APPI, a soft ionization technique which permits analysis of intact macromolecules including proteins and peptides. In MALDI, the sample is incorporated in a lattice of a crystalline matrix and irradiated by laser induces desorption and ionization of the sample/matrix mixture (Figure 4). The offline mode and insensitivity to sample impurities are two characteristic properties. MALDI possesses a benefit of independent analysis and possibility to re-analysis of the sample as well as permits analysis of on-target enrichments (paper IV). An additional feature in MALDI is the formation of almost exclusively singly charged ion species, which leads to a dramatic reduction in spectra complexity and easier data interpretation.
"Just don’t give up trying to do what you really want to do. Where there is love and inspiration, I don’t think you can go wrong"
Ella Fitzgerald

When hyphenating CE to MS one has to consider several aspects, such as the ionization technique used. Atmospheric pressure photoionization and atmospheric chemical ionization (APCI) have a tendency to be more mass flow sensitive than ESI. This indicates that injecting a large volume of sample will give a higher response in the MS. However, the low sample loading capabilities in CE becomes a limiting factor. The injection volume in CE is restricted to approximate 3% of the capillary or band broadening effects starts to occur. This indicates that the only possibility to increase the injection volume is to increase the inner diameter or increase the capillary length. On the other hand, APPI has a tendency to be more suitable for nonpolar compounds and BGEs with high salt content.

In protein and peptide analysis ESI/MS and MALDI/MS are the most commonly used techniques. Since MALDI operates under high vacuum, a challenge has been to transfer the solution from a liquid based separation to high vacuum. The method of choice has been an off-line system where a fraction collector has been used to spot down the liquid on a plate before analyzing with MALDI. However, due to the high efficiency of CE, large demands are put on the fraction collector.

Coupling MEKC with mass spectrometric detection has proven to be a great challenge. The main reason is that the most commonly used surfactant, sodium dodecyl sulphate (SDS) cause massive ionization suppression, contamination of the ion source and increased background signals when using ESI/MS. Nonetheless, there have been reports on the direct coupling of MEKC with ESI/MS utilizing SDS as a surfactant. Even though they could obtain the required limit of detection for the application, significant ionization suppression was found. Numerous attempts have been made to solve these limitations, such as the partial filling (PF) technique in combination with MEKC or reverse the migrating micelles to prevent the surfactants from ionizing and entering the ion source. These techniques therefore
require specific optimization, with large restrictions in the background electrolyte (BGE) composition. Other procedures reported have involved complicated capillary connections constructed to dilute or remove surfactants from the separation capillary effluent \cite{77-79}. Yet, other approaches have involved volatile surfactants or high molecular weight pseudostationary phases (PSP’s) \cite{80-82}. Despite these technical developments, coupling of MEKC with ESI-MS has so far been shown to require tedious and careful optimization while still suffers from limiting factors.

5.1 Tapered Capillaries

Tapered and narrow restrictors and capillaries have shown to aid in suppressing the bubble formation and to be an interesting alternative to frits in CEC \cite{6}. Tapering the capillary tip in CE has shown to be especially beneficial when working with low flow rates and with sheathless interfaces. The tapered capillary permit a more stable electrospray by making it possible to operate at lower ESI voltages and giving lower signal-to-noise ratios \cite{83}. The tapering of the tip can be made in different ways, like pulling during heat, etching with hydrofluoric acid or by mechanical grinding. The benefit of pulling is the simplicity and reproducible result. Unfortunately, the inner diameter changes giving rise to local changes in electric field. However, when using the etching procedure, the inner diameter can be protected. But storing capillaries can be problematic, since despite stopping the etching procedure, the process continues.

5.2 Requirements of the Interface

The interfaces for MS are originally designed to be used with higher liquid flows, commonly achieved with traditional HPLC. It is reasonable to believe that the ion source needs to be modified when hyphenating CE to MS due to the significantly lower volumes and its vulnerability to analyte losses and sample adsorption to capillary and ion source walls \cite{72, 84, 85}. This can be seen in Figure 5 from paper I where the signal intensity clearly benefits from a miniaturization.
Figure 5. Modifications of the interface can be necessary when hyphenating CE with MS. Paper I showed an improved signal intensity when moving the sprayer closer to the ionization region.

One of the major requirements of the interface is that the current from the CE is maintained or otherwise the separation will be interrupted. There are three main ways of securing the electrical circuit; liquid junction, sheath liquid interface, and sheathless interface (see Figure 6). In a liquid junction interface a stainless steel union serves as a point for the high voltage contact. A nebulizer gas and an extra liquid are introduced in order to create a stable spray. Even though this is an easy and excellent way to define the electric field some negative aspects exist. Due to the electrode reactions occurring inside the stainless steel union gas bubble formation could interfere with the separation and spray formation. The distance between the two capillaries and the sheath liquid/nebulizing gas tubing will create a small volume, leading to band broadening in the separation and the fact that the electric field is not defined at the end of the capillary will give rise to laminar flow. However, despite the mentioned challenges liquid junction is commonly used.
In paper I, II and V, a sheath flow interface was used. In this type of interface a sheath liquid and nebulizer gas is introduced at the tip of the capillary by concentric stainless steel tubes. This will thereby minimize the risk of gas bubble formations, avoid any post-column region with laminar flow and give a more robust interface with reproducible results. Studies have also shown an increase in theoretical plate numbers with the coaxial sheath liquid interface compared to the liquid junction interface. One other advantage with the coaxial interface over the liquid junction interface is that the sheath liquid is delivered independently of the BGE. This will provide more flexibility in the type of BGE used. [86, 87] With sheath flow interfaces the distance between the fused silica capillary and the stainless steel sprayer has a great impact on migration time, peak resolution and peak width as shown in paper I and Figure 7. The applied nebulizer gas creates a negative pressure at the tip introducing these side effects unless careful optimization is made.

The sheathless ESI interface does not introduce an extra liquid as with sheath liquid interfaces. Sheathless ESI uses a thin layer of a conductive material on the outside of the emitter end in order to secure the electric circuit. Even though several challenges arise with this method, such as instability of the conductive coating and the total reliance on the eluting solution to create the spray, sheathless ESI has shown a great potential.
Figure 7. Demonstrated in paper I, the capillary end position relative to the stainless steel sprayer tip has a great impact on migration time, peak resolution and peak width.

Figure 8. Shown in paper II, the added organic modifier to the BGE and sheath liquid has a large effect on peak intensities.
The discussion regarding the use of sheath liquid versus sheath less interface in ESI/MS has been debated for more than two decades. The main arguments have been that a sheath liquid setup would result in a dilution of the analyte. However, a sheath liquid might give a more stable spray due to the lowering in surface tension and a more robust system, and thereby avoiding organic solvents otherwise needed in the BGE. In paper V different sheath liquid flows where compared. Interestingly, larger peak areas, higher charge states of a monoclonal antibody, as well as a more stable spray were achieved with increased sheath flow (Figure 9). In paper II acetonitrile was added in both the background electrolyte and in the sheath liquid. This was made in order to control the interaction between the neutral analytes and nanoparticles. Figure 8 from paper II indicates that continuous full filling of nanoparticles in CE-MS could benefit from a sheathless ESI approach using a sheath liquid with low percentage of organic modifier in combined with a high amount in the BGE.

![Figure 9](image.png)

**Figure 9.** In paper V the sheath liquid flow was varied between 2 and 10 µL/min. The combination of highest peak intensity and fine structure gave an optimal condition at 8 µL/min. The sheath liquid consisted of ACN:MilliQ-water 50:50 (v/v).
The choice of metal coating onto the fused silica in sheathless ESI has a large influence. A silver layer have shown to be unstable under red-ox conditions at the ESI tip, and sputtered gold have a finite lifetime before the electrical contact is lost \cite{43, 48}. However, other coatings such as graphite-polyimide mixtures have shown a good performance and stability for a long time under oxidative stress \cite{46}, especially in combination with a tapered capillary. One major difference between sheathless ESI and sheath flow and liquid junction interfaces is the added organic modifier to the separation buffer to facilitate the ESI process. However, an organic modifier can be added to the BGE for other reasons than to obtain a stable spray. In paper II the organic modifier was added to vary the interaction between neutral analytes and the nanoparticles, and in paper III to precipitate serum albumin, aside from influence the protein-bead interaction. The type and amount of organic modifier have shown to largely impact the electrospray stability with both the sheath liquid and sheathless ESI interface. A careful optimization to avoid a decrease in EOF by too high concentrations or disrupting the electrospray process is therefore needed \cite{26, 88}.
6 Adaptation of Pseudostationary Phases to Mass Spectrometry

"Technology has to be invented or adopted"

Jared Diamond

On-column UV-detection is by far the most prevalent detection technique applied in CE and is also the most commercially available detection technique. MEKC was invented on such equipment and for this reason most of the pseudostationary phases in use today are UV-transparent. However, whereas coupling CE with MS detection was fairly straightforward, a number of drawbacks were identified. A major problem was the analyte ionization suppression, high background signals and contamination of the ion-source with non-volatile PSP’s \[^{29,74}\]. Three different solutions to solve these limitations will be discussed in this chapter. The first solution involved adaptation of the traditional MEKC protocols to MEKC/MS by adjustments of buffer composition, surfactant concentration, partial filling (PF), and reverse migrating (RM) PSPs. The second solution was to design new types of PSPs with improved MS compatibility by using high molecular weight and semivolatile PSPs. The third solution involved instrumental modifications including inventions in the electrospray interface and application of other ionization sources, such as APPI and APCI.
6.1 Micellar Electrokinetic Chromatography- Mass Spectrometry

The advantage of MEKC over HPLC is the higher efficiencies achievable as well as selectivity originating from both electrophoretic and chromatographic mechanisms. Furthermore, as the PSP is replenished after every separation, memory effects, stationary phase ageing and column clogging are minimized. However, the compatibility of MEKC with MS detection has, as previously mentioned, been poor. Still, studies have been conducted in which the buffer composition and surfactant concentration was altered to allow for MS detection \cite{54, 75}. Although the reported limit of detection was high, some analytes could still be detected. Ionization suppression, source contamination and high background signals were observed in all studies and the obvious solution to these issues was to exclude the surfactants from entering the ion source. Lamoree and co-workers \cite{89} suggested that coupling MEKC with MS detection can be realized by transferring the analyte zones to a capillary free of SDS by voltage switching. However, this technique requires a home built capillary coupling system. Furthermore, the efficiencies were about two-fold lower than for MEKC/UV, which was attributed to band broadening due to the application of a lower separation voltage and extra band-broadening in the coupling device. Nevertheless, the authors described a fascinating system for on-line heart cutting of sample zones in MEKC.

The first more general technique for rendering a standard MS compatible MEKC protocol was partial filling (PF) of the PSP (Figure 10.). With this technique only a section of the capillary at the injection side is filled with a PSP. Subsequently, the sample is injected and when a voltage is applied over the capillary the sample migrates through the PSP and reaches the detector ahead of the PSP. The analysis is then stopped before the PSP reaches the detector and thereby avoiding introduction of the PSP into the ion source. Drawbacks with the PF technique include the required introduction of additional variation with the extra injection of PSP. Furthermore, the electrolyte becomes discontinuous resulting in EOF variations between the PSP plug and the surrounding electrolyte, causing pressure variations and ultimately introduction of laminar flows \cite{90, 91}. Furthermore, the net velocity of the analytes is generally higher outside the PSP plug resulting in further band broadening. Another issue is that in the border between the PSP plug and the surrounding electrolyte the concentration of surfactants gradually drops, resulting in a micelle gradient due to diffusion and laminar flow effects.

Due to these drawbacks and the fact that the PSP plug generally is kept very short to avoid elution of surfactants into the ionization source, the resolution is always lower than for traditional MEKC \cite{92}. Method developments are therefore more complicated in PF-MEKC than in traditional MEKC.
Figure 10. (A) Schematic illustration of PF-EKC. Step 1: A plug of PSP is injected prior to injection of the sample. Step 2: As the voltage is applied, the analytes start to migrate through the PSP plug thereby becoming separated. Step 3: The analytes reaches the electrolyte phase prior to detection. The separation is stopped when all analytes have been detected and before the PSP reaches the detector. (B) Schematic illustration of RM-EKC. Step 1: The capillary is filled with PSP prior to injection of sample. Step 2: As the voltage is applied, the analytes start to migrate through the PSP and thereby becoming separated. The PSP migrates in the opposite direction into the inlet vial, thereby efficiently avoiding elution of surfactants into the ionization source. Step 3: The separation is stopped when all analytes have been detected.

In PF, the longest applicable plug length is obtained with a very low EOF allowing for a net reverse migration of the micelles. Thereby, the theoretical plug length would be equal to the total capillary length. Highly negatively charged PSPs with low pH electrolytes or neutral capillary coatings have made it possible to realize such long plug lengths and the technique have been named RM-EKC (described in Figure 10B) [77, 93]. Although some positively charged PSPs have been applied in reverse polarity for separation of negatively charged analytes, the technique is mainly applicable for positively charged analytes due to the very low or absent EOF in RM-EKC. However, RM is very similar to PF since the whole capillary is rarely filled with PSP due to the overwhelming risk of contaminating the ionization source. Despite the development of techniques that enable the use of traditional PSPs in EKC/MS, the counter ions of the PSP may still reach the detector causing ionization suppression. Thus, PSPs designed for use with MS detection (paper II) and innovations in MS detection are required.

6.2 Continuous Full Filling

Today, ESI is the dominating ionization technique used in liquid phase separations coupled to MS detection. ESI was originally developed for HPLC, and the performance of many of the earlier separations published on MEKC/MS were hampered because of unsuitable interfaces. Significant improvements in MEKC/MS were achieved as the ESI interfaces were miniaturized and adapted to the capillary format. Further improvement was ob-
tained with the orthogonal ESI-interfaces. These interfaces, spraying at an angle to the mass spectrometer inlet, had an improved tolerance to non-volatile electrolyte constituents [94]. However, further developments are required to realize the full potential of CE/MS [72, 84, 95]. Viberg [25] elegantly showed that nanoparticles could be used as a PSP in a continuous full filling (CFF) technique using an orthogonal ESI interface (Figure 11) in the same manner as in paper II. In CFF the PSP is suspended in the electrolyte similar to traditional MEKC. Thus, the electrolyte is continuous, avoiding the problems resulting from discontinuous electrolytes in e.g. PF-EKC. The introduced nanoparticles were excluded from entering the MS because of an orthogonal ESI interface. The particles could not, due to their larger momentum, deviate from the off-axis spray direction. If these highly concentrated nanoparticles in electrolyte suspensions would be sprayed on-axis several microgram per minute of non-volatile nanoparticles could risk entering the mass spectrometer. Several studies, mainly involving improvements in the nanoparticle based PSP, followed this proof-of-principle report [26, 96-102]. Later studies, including paper II, showed that CFF with nanoparticles could be performed without any negative effects on the analyte signal, minimizing contamination of the mass spectrometer and with efficiencies as high as one million plates per meter [26, 100, 101]. In paper II the nebulizer gas pressure and ionic strength were varied to investigate the effect on separation efficiency and contamination. Lowering the nebulizing gas pressure below 18 psi approximately fivefold increased the separation efficiency. However, no significant effect on separation efficiency or capacity factors was found when varying the ionic strength. Despite the lower nebulizing gas pressure no indications of nanoparticle contaminations inside the mass spectrometer could be found after months of usage.
Figure 11. A schematic picture showing the principle of CFF-CEC/ESI-MS from paper II, where the added nanoparticles to the BGE are transported through the open capillary. This will allow for a separation of neutral analytes based on the different partitioning of analytes to the nanoparticles. The electroosmotic flow is sufficiently high to generate a net transport towards the cathode, despite any anodic migration of the nanoparticles.
The number of publications involving CE and nanoparticle related techniques have increased exponentially during the past two decades (Figure 16). Despite developments of new PSP’s and new and modified ESI-interfaces, the full potential of EKC/MS may not yet have been realized. Recent developments in ionization techniques indicate that other ionization sources may be better suited for coupling of EKC with MS detection. Recently, two conceptually different ionization techniques were applied in MEKC/MS. Atmospheric pressure chemical ionization (APCI) and atmospheric pressure photoionization (APPI) applies electrical discharges and photons, respectively, to ionize the analytes in the gas phase. Interestingly, these two techniques were found to be virtually insensitive to the presence of surfactants in the BGE. Analogous to the improvements in ESI with orthogonal spraying, orthogonal APCI was also found superior to on-axis APCI [84]. However, although proven to possess an extensive potential in EKC/MS, ESI-MS is generally still more sensitive [84, 103-105].
Particles and beads are valuable separation media from a practical aspect, its high surface area and its versatility. The nanotechnology has become a promising field offering a potential for almost limitless range of applications. Due to the favorable surface-to-area ratio and its small size, nanoparticles have attracted scientists working especially in the field of separation science. Demonstrated in paper II neutral analytes where separated in CE-MS by introducing polymeric nanoparticles in the background electrolyte. They have shown great promises in off-target approaches by enriching and extracting specific analytes (paper III and IV). Magnetic nanoparticles or beads can easily be controlled via an external magnet and have the possibility be trapped on a surface and analyzed on-site.

Figure 12. In paper II nanoparticles where used to separate neutral analytes in CFF-CE/MS. Functionalized magnetic beads was used for enriching and separating low molecular weight proteins in an off-line approached demonstrated in the proof-of-principal paper III. However, shown in paper IV nanoparticles was used in the on-target enrichment study using titaniumdioxide and MALDI/MS.
7.1 New PSP’s with Improved Mass Spectrometric Compatibility

Two main approaches can be identified in the development of new PSPs with improved MS compatibility. On one hand, researchers have strived to reduce ionization suppression by developing high molecular weight PSPs with a CMC close to or equal to zero [81, 82, 106]. With these PSPs, accumulation of surfactants at the solvent-air interface is reduced causing less ionization suppression. Furthermore, electropherograms with less background signals in the low m/z-range are generated and the high molecular weight PSPs are in less extent ionized. Also, method developments are simplified as the electrolyte composition no longer affects the integrity of the PSP. On the other hand, reduced contamination of the ion source has been assessed by the use of semi-volatile surfactants [75, 107]. These two approaches are thus very similar to traditional MEKC. However, several new types of phases not falling into these categories have also been developed. Magnetic nanoparticles have been applied in a variety of analytical applications, such as in vivo examination via magnetic resonance imaging (MRI) [108] and in chip based systems (microfluidics) [108, 109]. The main characteristics of these particles are that they can be functionalized on the bead surface while still having the embedded magnetic properties allowing for manipulation of the particle with permanents magnets or electromagnets. Such magnetic beads have been applied in sample preparation and pre-concentration [110, 111]. In 1997 Rashkovetsky et al. [112] performed an enzymatic assay, affinity adsorption and isotachophoretic (ITP) focusing using magnetic beads in a CE system. In that application a small amount of magnetic beads containing immobilized antibodies was injected and trapped by a magnet in a neutral hydrophilic-coated fused-silica capillary. Subsequently, the sample containing the antigen was injected and the antigen was trapped onto the beads. The beads could then be washed and the antigen eluted by a change in the buffer pH. Finally, the separation voltage was switched on and ITP was performed.

An interesting application of nanoparticles was presented by Kan and Barron [113] and later by others [114]. In their application, poly(N-isopropylacrylamide) (PNIPAAm) modified temperature-sensitive latex beads were trapped in the capillary by an increase in the capillary temperature. Thereby, these particles could be used to trap analytes within the capillary. Lowering the temperature brought the beads back into suspension and the analytes could in that way be eluted from the capillary. These particles have also shown to be useful as coating in CE capillaries [113, 115]. The magnetic beads and the temperature sensitive latexes are not PSPs per se, but they do fulfill one of the requirements of a PSP, being a non-immobilized interaction phase. Even though these particles have not yet been applied in CEC/MS, they do present an interesting means of combining CEC with a non-trapped stationary phase with MS detection.
7.2 Magnetic Beads

The use of magnetic beads has gained in interest within the chemical and biochemical research. Magnetic beads are used in numerous applications including biosensors [116, 117], microfluidics and lab-on-a-chip platforms [118, 119], inorganic applications [120] and in immunoassays combined with capillary electrophoresis [121]. However, the use of iron oxide is not a new concept. The “father of medicine”, Hippocrates (460-370 BC), proposed that tumors should be cauterized by applications of hot iron. This principle is still valid even in modern time. Magnetic nanoparticles or magnetically tagged biological entities have the abilities to be manipulated and controlled with the use of magnetic fields. In this way these particles can be made to deliver e.g. anticancer drugs or radionuclide atoms to targeted regions such as tumors. Another attractive possibility with magnetic particles is to make magnetic metallic particles resonantly respond to a time-varying field leading to e.g. heated particles [122-125], which is related to the principle hyperthermia, that Hippocrates described 2400 years ago.

Robinson, Dunnill and Lilly showed the possibilities and benefits of using magnetic support materials and coated magnetic particles by purifying enzymes while still being able to retrieve the material from a liquid containing colloids and non-dissolved solids [126, 127]. Jean-Luc Guesdon and Stratis Avrameas showed in 1977 a further development of the principle by preparing a polyacrylamide-agarose bead with iron oxide added to the monomer mixture [128]. To perform an enzyme-immunoassay, a magnetic rack was constructed by mounting ten magnets on the side of a plastic rack with tubes being able to be put inside the rack next to the magnets. This way all washing solutions could easily be removed while still having the beads remained on the tube walls. With the magnetic bead setup time consuming centrifugation steps otherwise needed could thereby be avoided. Based on this work, Jean-Claude Antoine et al. showed the possibility to fractionate mouse and rat lymphoid cells using an insoluble support of polyacrylamide-agarose spherical bead with incorporated anti-mouse or anti-rat Ig antibody coated iron oxide particles [129]. They concluded that both adsorbed and non-adsorbed cells could be recovered between 80 and 100 %.
As mentioned, magnetic supports permit selective recovery of a catalyst in the presence of other suspended solids. However, several other advantages are a result of the magnetic separation in comparison with gravitational and centrifugal methods. In small scale applications, magnetic methods can speed up the total analysis time. Having embedded magnetic support entitles advantages such as the ability to be magnetically manipulated using permanent magnets or electromagnets while still being independent of microfluidic or biological processes. This advantage is used when separating using a microanalytical device such as a chip based or microfluidic technology. Electrophoretically mediated microanalysis (EMMA) is one example of the miniaturized microanalytical techniques based on a capillary format. In EMMA, reagent zones are merged electrophoretically without any dilution, turbulence or band spreading. One other advantage is the small dimensions of the CE system employed in EMMA which makes the technique useful in chip based setups.

On-target or on-plate sample enrichment strategies have been developed especially for enrichment of low volumes. The typical on-target enrichment strategy consists of a sample incubation step, washing and rinsing, and finally an addition of matrix before MS analysis. In paper IV an on-target enrichment and separation technique for multi- and mono-phosphopeptides was developed. The technique uses mesoporous titanium dioxide sintered onto a conductive glass slide and a technique analogous to thin layer chromatography. With this approach the enrichment, separation, and analysis are taking place on-target minimizing sample handling, risk of contaminations, and sample losses. A clear separation between multi- and monophosphorylated peptides for a more complex protein mixture at low concentration was demonstrated. Despite the advantages over other on-target enrichment methods the long separation time could be worrying with biological samples. However, the method has the potential to become a tool for comprehensive phosphoproteome profiling. An interesting approach would be to separate using electrophoresis on-target (Figure 13). Titanium dioxide has previously been introduced in CE and CEC to help separate e.g. analytes with similar structures and pKa values. By combining our method with an electrophoretic separation could possibly a great improvement in migration time and separation efficiency be accomplished together with the ability to analyze direct on-target.
Figure 13. The method used in paper IV could possibly be further developed into an on-target enrichment technique with an electrophoretic separation analyzed with MALDI.

7.3 Functionalized Magnetic Beads

Changes or structural modifications in protein expression has shown to be linked to both natural occurrences in the body and numerous of diseases [131-133]. The biomedical research field that has gained virtually an exponentially interest covers a large-scale study of the identity of proteins, concentrations, structures, functions and interactions is called proteomics [133-135]. As a result of the various peptide and protein degradation products released in the blood stream from a disease and the possibility to find diagnostic markers, the interest and applications in proteomics has virtually boomed [131, 132]. However, high abundant proteins are known to mask the measurable signal from other protein components present in a low concentration. It is therefore of great importance to have a reproducible method that selectively removes high abundant proteins such as human serum albumin, transferrin and immunoglobulin G. These three proteins are dominating in human plasma and represent more than 75 % of the total protein content [136]. Unfortunately, a removal of these high abundant proteins can result in co-removal of other proteins and peptides, an effect called the sponge effect [137, 138]. Therefore, a neat alternative to protein removal, and with its risk of sponge effects, would be to have a tool that selectively enriches low molecular weight proteins
while simultaneously discriminate larger proteins and thereby avoiding any co-removal. This is what has been investigated in paper III, where functionalized magnetic beads were used. The magnetic beads were designed to selectively enrich and fractionate low molecular weight proteins. The principle can be seen as a further development of the work of Jean-Claude Antoine et al. [129]. An inner layer with incorporated iron oxide particles will work as a cationic layer and will have the properties of enriching proteins from e.g. human plasma. In order to selectively fractionate high and small molecular weight proteins, an outer layer consisting of agarose-dextran mixture will function as a mesh hindering larger proteins from penetrating the layer, and thereby be enriched in the inner cationic layer (Figure 14). Four different functionalized magnetic beads with varying molecular weight cut-off was investigated. In combination with testing different bead compositions, sample preparation protocols using different pH and organic content were evaluated. A significant reduction of high abundant proteins (serum albumin), desalting and a simultaneously enrichment of low molecular weight proteins was demonstrated. The functionalized magnetic beads showed a promising use in biomarker discovery or in proteomics using human body fluids.

Figure 14. A schematic illustration from paper III showing how high molecular weight proteins are hindered from penetrating the outer dextran layer. In contrast, the low molecular weight proteins are able to penetrate and thereby be enriched by the inner cationic layer. Iron oxide is incorporated in the beads which makes them easily controllable via a magnet. The magnetic properties facilitate enrichments of low molecular weight proteins by enabling easy washing steps, minimizing carry overs and high throughput possibilities.
7.4 Antibodies and Immunoaffinities

One variation of traditional MALDI is immunoaffinity MALDI (iMALDI) \cite{139-141}. Antipeptide antibodies are immobilized on an affinity bead and mixed with isotopically labeled peptides and the digested proteome of interest. After incubation and washing, the bead-containing immunoadsorbed peptides could be arranged on a MALDI target plate. The added MALDI matrix solution enables elution of affinity bound peptides from the immobilized antibodies. The iMALDI will thereby permit MALDI analysis of the peptides with a high sensitivity and specificity of target molecules with quantitative possibilities. Successful qualitative and quantitative studies of complex mixtures will however continue to be dependent of appropriate cleaning, fractionating pre-analysis steps or on appropriate extraction methods (paper II-IV). In addition to nanoparticles, functionalized magnetic beads and pseudostationary phases in CE on-line immunoaffinity capillary electrophoresis (IACE) have emerged as a useful two-dimensional analytical technique. The method permits online capture of a targeted molecule on a solid support and released by changing the composition of the running buffer followed by separation by CE. Rashkovetsky et al. \cite{112} demonstrated how sheep Ig covalently coated magnetic beads was used to capture a mouse monoclonal antibody in a neutral coated fused silica capillary. After capture, the mouse antibody was released, preconcentrated by isotachophoresis and detected by UV detection. Chen et al. \cite{121} showed how protein A-coated magnetic beads where trapped into a neutral coated capillary as a support for an antibody of interest. The corresponding antigen was thereafter immunocaptured and preconcentrated online. By transient ITP and CZE, the antibody and antigen could be stacked and separated - resulting in a distinct advantage of simplicity and automatic preparation of immunoaffinity support that can be renewed after each analysis.
Figure 15. Paper V showed the importance of capillary temperature when analyzing a monoclonal antibody. With higher temperatures less adsorption to capillary walls and higher signal intensities can be achieved.

Monoclonal antibodies (mAbs) are a group of biotherapeutic drugs that has shown progress as disease indicator in for example cancer, immunological disorders and Alzheimer’s disease [142-144]. The use of antibodies in IACE could thereby limit sample extraction, cleanup and even enrichment due to its selectivity. However, mAbs are generally quite complex with its large size, multi-chain structure, and protein folding. To make the situation even more complex monoclonal antibodies has shown heterogeneities in forms of glycolysilations, deamidations, oxidations etc. [145, 146]. It is therefore of major importance to be able to separate and characterize monoclonal antibodies and even desalting the sample with a universal method without tedious method development. This was demonstrated and investigated in paper V where, to our knowledge, the first study of intact monoclonal antibody using non-coated capillary electrophoresis hyphenated with mass spectrometry was presented. The key parameter to be able to analyze mABs using CE-ESI/MS is to minimize the adsorption to capillary walls (Figure 15). When combining the result from the different capillary temperatures with corresponding CE currents a clear difference can be visualized. At lower temperatures the current breaks in comparison to temperatures above 40 °C. However, raising the temperature up to the highest possible with the used instrument gave an even higher signal intensity. This indicates less adsorption and higher charged sites with temperatures as high as 60 °C.
8 Concluding Remarks

"Everything should be as simple as it is, but not simpler"

Albert Einstein

Indicated by all the number of published articles (Figure 16), CE and CE-related variations is a technique that is getting more attention. However, more research is needed to fully use their potentials. The use of MS friendly pseudostationary phases is one reason for the interest in CE related techniques and will hopefully become even more appreciated in important fields such as in clinical applications. The use of nanoparticles in sheathless ESI has shown to be an excellent way of combining ESI friendly pseudostationary phases with the good properties of the sheathless interface. Since CZE is unable to separate neutral analytes, the use of pseudostationary phases is an excellent way of combining the high efficiency achieved in CE with the selectivity in HPLC without any drawbacks such as memory effects or clogging of the stationary phases. As paper II indicates, the new ESI/MS friendly pseudostationary phases could be a good way of solving limitations with complex matrixes using CE-ESI/MS without tedious modification steps or losing sensitivity because of ion suppression. The study also showed the importance of the organic modifier added to the BGE and nanoparticle mixture in capillary electrophoresis. However, the choice of organic modifier and amount of acid added to the background electrolyte has shown to have an effect on the ionization. This phenomenon and the effect of other important parameters on a monoclonal antibody, such as the temperature and sheath liquid flow rate, has shown to be of major importance for system stability, sample losses, ionization and in the end detecting your analyte of interest (paper V). Since samples from clinical applications normally are very complex and contains a high amount of salt, nanoparticles as a pseudostationary phase in CE-APPI/MS could however be the method of choice due to the more forgiving ion source regarding salt content.

In paper I the APPI/MS interface was investigated in order to be optimized for the use with capillary electrophoresis. Despite the different ionization techniques in APPI and ESI the same CE spray needle can be used. Paper I indicated that the protruding capillary end from the stainless steel sprayer and the nebulizing gas flow had a large influence on the detection
sensitivity and should be optimizing accordingly. It could also be concluded that the available APPI interface is not fully optimized for CE-MS. However, small modifications and miniaturization of the interface, as well as changing the capillary inner diameter are some ways to optimize the conditions for CE-MS. Although changing ion source and optimizing MS parameters could be a good way to improve the detection, a sample preparation step is in most cases necessary. One way is to use a functionalized magnetic bead (paper III) that could fractionate interfering molecules while simultaneously enrich molecules of interest. The benefits of having a magnetic bead are many, like the possibility of minimizing carry-over effects due to easy handling, and the ability to automate the whole sample preparation procedure just to mention a few. Another way could be to use an on-target enrichment and separation approach and selectively ionize the analytes and target of interest using e.g. MALDI (paper IV).

**Figure 16.** The number of published articles with a topic concerning (a) CE and the sum of (b) nanoparticle based CE techniques (source ISI Web of knowledge).

This thesis has covered how complementary separation techniques could be used in combination with mass spectrometry. One important aspect with all methods and their optimizations has been the possibility to automate and thereby be able to work with large sample sets normally present in clinical applications. Capillary electrophoresis has during the last decade matured and can now in an easy manner be coupled to an MS instrumentation and separate in more than one dimension, e.g. CE using a pseudostationary phase. With nanoparticles or functionalized magnetic beads as a static or pseudostationary phase in a commercial CE, an interesting pre-fractionator similar to IACE could be achieved. The possibility to further develop CE and the ideas of being used in other areas of interest has been highlighted in this doctoral thesis. Hopefully will CE and its variations hyphenated to MS be considered as an excellent alternative to more conventional techniques in future studies.

Masspektrometri (MS) har under de senaste 25 åren fått ett enormt genomslag tack vare sin höga känslighet, specificitet och låga detektionsgränser. MS används rutinmässigt t.ex. inom det viktiga vetenskapsfältet proteomik [16] och uppmärksammades 2002 med ett Nobelpris för två jonisationstekniker; elektrospray jonisation (ESI) samt laser desorbtions jonisation (LDI) vilka är illustrerade i figur 4.
I och med de stora framgångarna med masspektrometri som separationsmetod, var det naturligt att vilja koppla samman CE med MS. Dock uppstod problem med att kunna sluta den elektriska kretsen utan att påverka separationen eller jonisationen.

Figur 17. I denna doktorsavhandling diskuteras hur alternativa separationstekniker, så som kapillärelektrofores, funktionaliserade magnetiska partiklar och nanopartiklar kan utnyttjas i kombination med masspektrometri.

Denna doktorsavhandling syftar till att kunna koppla samman alternativa separationstekniker, så som elektrofores, nanopartiklar och magnetiska partiklar med masspektrometri (Figur 17). Avhandlingen är baserad på fem studier. I artikel I studerades hur en fotojonisationskälla (APPI) kunde optimaliseras för kapillärelektrofores. Utfallet av studien visade på att en mindre jonkälla bör tillverkas samt att en optimering av distans mellan kapillär och sprynål är avgörande för injektionsvolymer, breddning av toppar samt spray-stabilitet (se även Figur 5 och Figur 7). I artikel II undersökt en pseudostationär fas bestående av polymera nanopartiklar med en kontinuerlig påfyllningsteknik. Trots att jonisationen skedde via ESI kunde samma erfarenheter och slutsatser användas från första studien i och med att samma typ av sprynål använts (Figur 6, sheath liquid). I studien undersökt bland annat hur separationen, jonisationen, repeterbarhet samt detektionsgränsen kunde optimaliseras och förbättras genom att exempelvis variera mängden organisk modifierare tillsatt i bufferten. En annan typ av partikel användes i artikel III, en funktionaliserad magnetisk partikel (Figur 14). Den grundläggande principen för denna partikel är att kunna fraktionera de mest förekommande komponenterna i komplexa prover och samtidigt anrika de som förekommer i låg koncentration. Detta är viktigt framför allt i sökande av biomarkörer inom vetenskapsområdet proteomik. Att det går att anrika och
separera proteiner och peptider på ett annat sätt visades i artikel IV genom en tredje typ av partikel. Ett tunt lager av titaniumdioxid sintrades på en konduktivt ledande glasskiva där enkel- och flerfosforyllerade peptider separerades och detekterades med en variant av tunnskiktskromatografi (TLC) tillsammans med matris-assisterad laserdesorption/jonisations (MALDI) masspektrometri.

10 Acknowledgments

"As we express our gratitude, we must never forget that the highest appreciation is not to utter words, but to live by them"

John F. Kennedy

There are many I would like to show my appreciation, especially my **family** and **friends** for all your love and support! **Mamsen**, jag vet inte hur ska ska beskriva vilken stor inspiration du är! Dock finns det en kort berättelse som skämtsamt beskriver all den kärlek och engagemang du ger mig och Henrik:

_A man calls his mother._

“Mom, how are you?”

“Well, not to good”, says the mother. “I’ve been very weak.”

_The son says, “Why are you so weak?”_

_She says, “Because I haven’t eaten in 38 days.”_

_The son says, “That’s terrible! Why haven’t you eaten in 38 days?”_

_The mother answers, “Because I didn’t want my mouth to be filled with food if you should call”_


I would like to show my biggest gratitude to my supervisors, **Jonas Bergquist, Magnus Wetterhall** and **Charlotta Turner**. I will always be extremely grateful for giving me this opportunity to pursue as a Ph.D. student and guiding me into the world of science. “Dojjan”, your enthusiasm and inspiration has occasionally been a catcher in the rye.

I would especially show my appreciations to my dear friends **Marcus Sjödin** and **Mattis Fondell**. Marcus, you have given me a great time with many laughs both at and outside work - one can’t ask for a better friend, office mate and colleague! Mattis, it is thanks to our friendship and your support I got through my undergraduate studies!
This work couldn’t have been possible without Matthias Pelzing, Peter Spégel, Jakob Axén, and all my other excellent collaborators! Thank you for all the scientific discussions and practical help you all have given me. Matthias, I would in particular show my appreciations to you and your family. I felt so warmly welcomed and learned extremely much about CE-MS during my Aussie-German days, and I will never forget the “exploding lamb”.

I would also like to give many thanks to Nanosep Analytical AB and GE Healthcare Uppsala for giving me this opportunity to work with your nanoparticles and beads.

Thank you all at the department, in particular Jörg Hanrieder, Sofia Lindahl and Erik Petersson for your support and happy spirits.

Last but not least, my girlfriend Lotten! Even though our journey has just begun I can see a lovely and happy future together!
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