Nanoparticles in Capillary Electrophoresis hyphenated with Mass Spectrometry

-What are the benefits?

David Malmström

Department of Physical and Analytical chemistry,
Analytical Chemistry
Uppsala University

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Abstract

Capillary electrophoresis is a technique that separate compounds based on charge and size. During the last two decades capillary electrophoresis (CE) and mass spectrometry (MS) has gained in interest, become more robust to use and able to separate neutral analytes. The separation of neutral analytes was first achieved with packed columns, but several disadvantages can be obtained with a stationary phase and the method of packing capillaries. Therefore, pseudostationary phases became a good alternative. The risk of clogging, memory effects and lower efficiency could be minimized with pseudophases. However, since mass spectrometry has become the most important analytical detector, and play a key role in the search for biomarkers in clinical applications, it is important that CE can successfully be combined with MS. To obtain this hyphenation several types of interfaces for the vital ion source exist. In paper I an atmospheric pressure photoionization interface was investigated in order to accomplish an improved detection sensitivity. The knowledge attained with this type of interface could then be transferred to the one used in paper II, the electrospray ionization interface (ESI), where the use of a MS friendly nanoparticle based pseudostationary phase was investigated. Both studies showed that it is still possible to improve the separation technique and modify the ion source in order to improve the detection sensitivity for capillary electrophoresis hyphenated with mass spectrometry.

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

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"Things should be made as simple as possible, but not any simpler"

Albert Einstein
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 and took part in writing the paper.

Paper II: Planned the study, performed the experiments and wrote the paper.

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Papers not included in this thesis


† These authors contributed equally to this manuscript
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## Abbreviations

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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>APPI</td>
<td>Atmospheric Pressure Photoionization</td>
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<tr>
<td>APCI</td>
<td>Atmospheric Chemical Ionization</td>
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<tr>
<td>BGE</td>
<td>Background electrolyte</td>
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<tr>
<td>CFF</td>
<td>Continuous Full Filling</td>
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<td>CE</td>
<td>Capillary Electrophoresis</td>
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<td>CEC</td>
<td>Capillary Electrochromatography</td>
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<tr>
<td>CMC</td>
<td>Critical Micelle Concentration</td>
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<td>CZE</td>
<td>Capillary Zone Electrophoresis</td>
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<td>EKC</td>
<td>Electokinetic Chromatography</td>
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<tr>
<td>EOF</td>
<td>Electroosmotic Flow</td>
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<tr>
<td>ESI</td>
<td>Electrospray Ionization</td>
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<tr>
<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
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<tr>
<td>i.d.</td>
<td>Inner dimension</td>
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<tr>
<td>ITP</td>
<td>Isotachophoresis</td>
</tr>
<tr>
<td>MALDI</td>
<td>Matrix Assisted Laser Desorption/Ionization</td>
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<tr>
<td>MEKC</td>
<td>Micellar Electrokinetic Chromatography</td>
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<tr>
<td>MS</td>
<td>Mass Spectrometry</td>
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<tr>
<td>MS/MS</td>
<td>Tandem Mass Spectrometry</td>
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<tr>
<td>o.d.</td>
<td>Outer dimension</td>
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<tr>
<td>PF</td>
<td>Partial Filling</td>
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<tr>
<td>PSP</td>
<td>Pseudostationary Phase</td>
</tr>
<tr>
<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 Hjertén 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 conventional high pressure liquid chromatography (HPLC) materials 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) that reduces 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 can lead to column-to-column variation. This will affect the EOF since it is controlled mainly by the surface of the packing material. Due to the difficulties in the packing of the capillaries and thereby varying EOF CEC can be seen as irreproducible in a larger perspective. There have been many different way of improve 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, the development in CEC is still active and developing since its breakthrough in the second half of the 1990’s as can be seen in Figure 1.
Another way to circumvent the limitations of packed columns was to use a pseudophase. This interesting technique was introduced by Terabe and others in 1984 where a solution of ionic micelles was introduced in the background electrolyte [10-14]. This separation principle first mentioned by Nakagawa [15] and is based on that micelles can migrate in an aqueous solution by electrophoresis.

The use of mass spectrometry (MS) has during the past 25 years reached an outstanding position among analytical methods due to its sensitivity and low detection limits. One of the areas where mass spectrometry has become the method of choice is in the enormous field of complex protein samples – proteomics [16]. MS has also been recognized by the Nobel Committee by giving the Nobel Prize in chemistry in 2002 for the ESI and laser desorption ionization. Olivares et al. [17] and Smith et al. [18] were the first to combine the powerful technique of mass spectrometry with capillary electrophoresis. The interface was based on the electrospray ionization (ESI) technique 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].

During the last decade, nanoparticles have been a hot topic, including as PSP in CE (see Figure 10). The use of nanoparticles as PSP’s, in combination with continuous full filling (CFF) and an orthogonal ESI-interface, has shown to be a good way to separate neutral analytes without affecting the electrospray process or contaminate the ion source [24, 25]. An orthogonal method of coupling MEKC and MS utilize an alternative ionization source – atmospheric pressure chemical ionization (APCI) and atmospheric pressure photoionization (APPI) [26]. In these methods the ionization efficiency has been shown to be unaffected by the surfactant SDS using both APCI and APPI [26-28].
This licentiate thesis is based on fundamental studies showing how the separation as well as the coupling of CE with MS can be improved. **Paper I** shows how 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 would be favorable. In **paper II**, a pseudostationary phase involving nanoparticles and the continuous full filling technique was investigated. Both separation and ion source parameters were investigated and optimized leading to improvements in column-to-column variability and detection limits.
2. Capillary Electrophoresis

Capillary electrophoresis (CE) is an umbrella term for many different methods. The general benefit of CE is the highly efficient separations for all the electrophoretic driven separations in CE. However, the inability to separate neutral analytes with traditional capillary zone electrophoresis (CZE) is a major limitation. But capillary electrochromatography (CEC), which is a packed capillary with the same stationary phase as in HPLC, could solve this challenge. Although CEC and HPLC are very similar, the main difference is the flat flow velocity profile generated by the EOF 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. But unfortunately, the reproducibility was a major challenge in CEC. The problem turned out to depend 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 remain a challenge [9].

In the predecessor of capillary electrophoresis, gel electrophoresis, the gel was a good way of avoiding joule heating, but unfortunately lead 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 has also been investigated, similar to traditional gel electrophoresis. However, producing and using gel filled capillaries was often quite difficult due to bubbles present inside the gel. The challenge was elegantly solved by using non-cross-linked gels because the gel could be injected into the capillary and after separation discarded [29, 30]. Apart from the non-cross linked acrylamide gels, other types of non-trapped stationary phase have been used in CE and CEC (and will be discussed in chapter 3).
2.1. General concept of Capillary Electrophoresis

The separation by electrophoresis is based on different movement of charged compounds (ions) by attraction or repulsion in an electric field [2, 3]. In CE, the electrophoretic separation is taking place in a thin glass capillary made of fused silica. The inner diameter of the capillary is typically 25-75 μm, which can be compared to a human hair shaft, typically at 100 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 connect the applied high electric field. In gels this applied current is in higher degree limited by Joule heating, the heating 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 potential. Electric fields between 100 and 500 V/cm is possible to use with only minimal heat generation. With higher electric fields come shorter analysis time, higher peak efficiencies and better resolution. The inner diameter (i.d.) of the capillary also has a large effect on the limitations. An increase in i.d. lead to a lowering of the surface-to-volume ratio. A capillary with 50 μm in i.d. would 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 for very efficient dissipation of Joule heat generated from large applied electric fields. As a result, electrophoretic separations in capillary electrophoresis can easily be performed at up to 30 000 volts.

The simplest form but also the most commonly utilized type of CE is capillary zone electrophoresis (CZE). The capillary is filled with an appropriate separation buffer with a desired pH, sample is injected in the inlet (normally the anode end) while the other end of the capillary is grounded either by placing it in another buffer vial or adding an extra liquid flow (sheath liquid) (see Figure 2). 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 were the only driving force acting on the ions, just net positively-charged compounds (cations) would be separated along the capillary while neutral compounds would remain static and net negatively-charged compounds (anions) would be driven back into the buffer vial in the inlet end, making CZE quite limited in use. Fortunately, a force called electroosmotic flow (EOF) creates a movement of all components in the capillary towards the cathode (under normal conditions).
Figure 2. The principal of CE where the negatively charged silica surface in combination with the applied electric current creates the EOF indicated with a blue arrow. Analytes will be migrating with an electrophoretic mobility determined by their charge and mass which then will lead to a separation of the analytes. On the capillary wall the static Stern Layer can be seen, as well as the moving Outer Helmholtz Plane in the middle of the capillary.

A marker is useful to add as an internal standard in order to determine relative migration times for the compounds. A neutral compound, e.g. dimethyl sulfoxide (DMSO) is used as only charged species can be separated in CZE while neutral analytes will migrate with the EOF. Herein lay also one major problem with CZE, which lead to the development of electrokinetic chromatography, EKC, including micelles and nanoparticles.

2.2. Electroosmotic Flow

The silanol groups (SiO⁻) on the inside wall of the capillary (see Figure 2) will attract cationic species from the buffer. This attracted ionic layer has a positive charge that will decrease exponentially as the distance from the capillary wall increases. The double layer that is formed closest to the surface is named Stern Layer or Inner Helmholtz [31] 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 but 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 pumping mechanism that will carry all analytes. A flat flow front profile will be created by the EOF in contrast to a parabolic flow created by a pressurized driven flow. Due to the flat flow profile all analytes will be affected in the same way leading to sharp peaks in contrast to the parabolic flow typically present in HPLC. The 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. By optimizing the EOF it is then possible to have a short 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 creating a problem in protein and peptide analysis due to low efficiencies, low recovery, poor reproducibility, and decreased sensitivity [32, 33]. Many ways of modifying the capillary in order to improve the separation and sensitivity have therefore been tried, such as extreme pH values, high salt concentrations and different additives into the BGE [34-38]. However, when using ESI/MS the preferable strategy has been to permanently coat the inner capillary wall. The permanently coatings are attached to the capillary wall prior to analysis and are therefore excluded from the buffer electrolyte making it suitable with ESI/MS. However, despite the regeneration of the coatings by flushing the capillary in between injections with a diluted solution in order to overcome stability issues and repeatability, the time and cost required to prepare the coating could be a challenge to overcome. Coatings could be used in order to reduce, neutralize or reverse the EOF as well. Gilges et al. [39] showed for instance how polyvinyl alcohol and other polymers could be used as a dynamic coating and effectively suppress the EOF and thereby focus the different zones, as in isoelectric focusing (IEF). Except modifications on the inside of the capillary wall, coatings on the tip and outside wall in order to secure the electrical connection between the capillary end ESI source using metalized coatings and emitters has been developed [40-46]. This type of modification has shown an important role in sheathless ESI, which will be discussed in chapter 5.2.
3. Electrokinetic chromatography

“Never, never, never give up”

Winston Churchill

Since CZE cannot separate neutral analytes due to the lack of electrophoretic mobility, the technique Electrokinetic chromatography (EKC) was developed. In 1982 Terabe and co-workers [47] published the first successful separation with sodium dodecyl sulfate (SDS) as an additive in the buffer. The technique was later called 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 in order to use mass spectrometry as detection without ion suppression and ion source contamination.

3.1. Why Pseudostationary Phases?

The most frequently used separation technique for analysis in liquid phases is HPLC. However, since it requires large sample and buffer volumes, miniaturized μLC and nanoLC systems [48, 49] have gained in popularity. To improve the separation efficiency, the particle size in the stationary phase has been decreased in parallel to the instrumentation miniaturization [50]. Due to the nature of a stationary phase the risk of sample adsorption and column clogging could get higher with the decreased column- and particle size. The problem with memory effects in the column due to “sticky samples” is another problem existing 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 [47, 51].
3.2. Micellar Electrokinetic Chromatography

Terabe was one of the pioneers in introducing selectivity in CE with the technique electrokinetic chromatography [10-14]. However, it was later called micellar electrokinetic chromatography (MEKC) to specify the use of micelles. These initial reports by Terabe and co-workers resulted in an explosion of studies during the 1990’s with the use of different surfactants, polymers, macromolecules and particles as PSP’s [14, 47, 52, 53]. The technique was based on partitioning analytes between two phases, a pseudostationary phase composed of charged micelles and the surrounding electrolyte phase migrating with 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 out of the capillary the micelles will migrate towards the detector due to the high EOF. Analytes would be portioned 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 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.* 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.
By adding SDS to the BGE, anionic, cationic, and neutral analytes can be separated 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 [54] or mixing different surfactants. However, the surfactant concentration, buffer, pH of electrolyte solution, or the temperature are other parameters to manipulate in order to vary the selectivity. But the most convenient way is to incorporate or modify an organic solvent to the BGE. That was also shown by S.K. Poole and C.F. Poole [54, 55] to be the most efficient way to manipulate the selectivity. The buffer composition and concentration, pH, temperature, and voltage only had a small influence on selectivity [56].

Coupling MEKC with a mass spectrometric detection has proven to be a great challenge. The main reason is due to 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 [28, 57]. Nonetheless, there have been reports on the direct coupling of MEKC with ESI/MS utilizing SDS as a surfactant [27, 58]. Even though they could obtain the required LOD for the application, significant ionization suppression was found. Numerous attempts have been made to solve these problems, such as the partial filling (PF) technique in combination with MEKC [59] or reverse the migrating micelles to prevent the surfactants from ionizing and entering the ion source.
4. Mass Spectrometry

"Intelligence is the ability to adapt to change"

Stephen Hawking

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 the analyte [60-64]. 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 given about the 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. [65, 66]
4.1. The use of Mass Spectrometry

Mass spectrometry has gained an enormous impact in important fields such as proteomics, peptidomics, and metabolomics [67, 68]. This is due to the introduction of 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 [69], MS has been used in other fields such as forensic science. The use of gas chromatography/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 early 1960s and have returned information about the solar wind isotopes and other isotopic ratios. [66] Since MS can be used in so many different fields of interest, high demands are put on the instrumentation. The MS 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 1963, Richardson [20] developed a technique where he 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 et al. ESI as well as thermospray (TS) and aerospray (AS) all share the same basic principles. 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 can be repeated until ions exist in ambient gas. The electric field will let the droplets and ions migrate towards the inlet of the MS.
Figure 4. Two commonly used interfaces in MS; to the left is the APPI ion source with the photoionization lamp seen in the window underneath the heater. To the right is the ESI interface with the spray needle tip visible at the entrance to the mass spectrometer.

One of the latest among the soft ionization techniques in mass spectrometry is the APPI ion source [70]. This ionization technique has shown less dependency on buffer composition compared to the ESI source, and works good at low flow rates. This indicates that APPI would be suitable with CE, due to the commonly used non-volatile buffers and low flow rates [71]. 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 can a so-called dopant be introduced. This was shown by Sprangler et al. [72] in where introducing a suitable substance could significantly increase the number of ions. 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).
5. Modifications when hyphenating Capillary Electrophoresis to Mass Spectrometry

When hyphenating CE to MS one has to consider several aspects, such as the ionization technique used. APPI and APCI, for instance, 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 MS. However, the low sample loading abilities in CE becomes a limitation. The injection volume in CE is limited to approximately 3% of the capillary, leading to that the only possibility is to increase the inner diameter or increase the capillary length in order to increase the injected sample volume. On the other hand, APPI has a tendency to be more suitable with 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, transferring solutions from a liquid based separation to the high vacuum in the ion source has been a challenge. 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 a mass spectrometric detection has proven to be a great challenge. The main reason is due to 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 [28, 57]. Nonetheless, there have been reports on the direct coupling of MEKC with ESI/MS utilizing SDS as a surfactant [27, 58]. Even though they could obtain the required LOD for the application, significant ionization suppression was found. Numerous of attempts have been made to solve these problems, such as the partial filling (PF) technique in combination with MEKC [59] or reverse the migrating (RM) 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 [73-75]. Yet, other
approaches have involved volatile surfactants or high molecular weight pseudostationary phases (PSP’s) [76-78]. Despite these technical developments, coupling of MEKC with ESI-MS has so far been shown to require careful optimization and suffer from limitations in selecting interaction phase.

5.1. Tapered capillaries

Tapered and narrowed restrictor capillaries have shown to be an aid in suppressing the bubble formation and an interesting alternative to frits in CEC [6]. Tapering the capillary tip in CE has shown to be especially good when working with low flow rates and with sheathless interfaces. The tapered capillary permit a more stable electrospray and make it possible to operate at lower ESI voltages, and give better signal-to-noise ratios [79]. The tapering of the tip can be made in different ways, like pulling during heat, etching with hydrofluoric acid or by mechanical grinding. However, to reproduce a tapered capillary tip by mechanical grinding could be a challenge.

5.2. Requirements of the interface

The interfaces for MS are originally designed to be used with higher liquid flows, commonly achieved with HPLC. Since CE uses significantly lower volumes and losses of analytes to the walls due to adsorption will be more crucial, it is therefore reasonable to believe that the ion sources need to be modified. This has been indicated in several studies and shown as well in paper I [71, 80, 81].

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 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 could be introduced in order to create a good spray. Even though this is an easy and excellent way of 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. Despite the mentioned challenges and the fact that the electric field is not defined at the end of the capillary, leading to a laminar flow beyond that point, liquid junction is commonly used.

Figure 6. Three variations of ESI needles for CE-MS: liquid junction (left), sheath flow (top), and sheathless interface (right).

In both paper I and II, a sheath flow interface was used. In this type of interface the sheath liquid and nebulizer gas is introduced at the tip of the capillary by concentrically stainless steel tubes. This will thereby minimize the risk of gas bubble formations, avoiding any post-column region with laminar flow, and give a more robust interface and 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. [82, 83] However, since sheath liquid is introduced, a dilution of the sample is unavoidable.

The sheathless ESI interface on the other hand does not dilute the sample since no extra liquid is introduced. 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. The choice of metal coating onto the fused silica 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 [41, 46]. However, other coatings such as graphite-polyimide mixtures have shown a good performance and stability for a long time under oxidative stress [44] 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 in order to facilitate the ESI process. However, an organic modifier can be added to the BGE for other reasons than facilitate a good spray. In paper II the organic modifier was added in order to vary the interaction between neutral analytes and the nanoparticles. 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 [25, 84].

Figure 7. Paper II showed that the amount of organic modifier in the BGE and the sheath liquid had a large affect on the ionization efficiency.
6. Adaptation of Electrokinetic Chromatography and Pseudostationary Phases to Mass Spectrometry

On-column UV-detection is by far the most prevalent detection technique applied in CE and it is also the detection technique with which most of the commercially available CE instruments are equipped. MEKC was invented on such equipment and for this reason most of the PSPs in use today are UV-transparent. However, whereas coupling of CE with MS detection was fairly easy, a number of problems were identified. A major problem was the analyte ionization suppression, high background signals and contamination of the ion-source with non-volatile PSP’s [28, 57]. Three different solutions to solve these problems will be discussed in this chapter. The first solution involved adaptation of the traditional MEKC protocols to MEKC/MS. This involved adjustments of buffer composition, surfactant concentration, partial filling (PF) of the PSP, and reverse migrating (RM) PSPs. The second solution was to design new types of PSPs with improved MS compatibility. This involved high molecular weight as well as semi-volatile PSPs. The third solution involved instrumental modifications including inventions in the electrospray interface as well as application of other ionization sources such as APPI and atmospheric pressure chemical ionization (APCI). Inventions in MEKC/MS within these three categories will be reviewed in the following sections.
6.1. Traditional Micellar Electrokinetic Chromatography adapted to Mass Spectrometric detection

The important advantages of MEKC over HPLC are 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 were conducted in which the buffer composition and surfactant concentration was altered to allow for MS detection [52, 58]. Although the LODs reported were 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 problems was to exclude the surfactants from entering the ion source. Lamoree and co-workers [85] suggested that coupling of MEKC with MS detection could 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 heartcutting of sample zones in MEKC.

The first technique being more general for rendering a standard MEKC protocol MS compatibility was PF of the PSP (Figure 8). 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, thereby avoiding introduction of the PSP into the ion source. Drawback with the PF technique includes 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 [86, 87]. Furthermore, the net velocity of the analytes is generally higher outside the PSP plug resulting in further band broadening. Another problem is that in the border between the PSP plug and the surrounding electrolyte the concentration of surfactants gradually drops due to diffusion and laminar flow effects ultimately resulting in a micelle gradient.
Due to these problems and due to 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 [88]. Obviously, method development is therefore more complicated in PF-MEKC than in traditional MEKC.

Figure 8. (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 reach 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 would be realized 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 length and the technique has been named RM-EKC (described in Figure 8B) [73, 89]. Due to the very low or absent EOF in RM-EKC, the technique is mainly applicable for positively charged analytes, although some positively charged PSPs have been applied in reverse polarity for separation of negatively charged analytes. However, due to the risk of contaminating the ionization source the whole capillary is rarely filled with PSP and therefore in practice very similar to PF. 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 and innovations in MS detection are required.
6.2. New Pseudostationary Phases with improved Mass Spectrometric compatibility

In the development of new PSPs with improved MS compatibility two main approaches can be identified. 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 [77, 78, 90]. With these PSPs, accumulation of surfactants at the water-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 development is 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 [58, 91]. 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) [92] and in chip based systems (microfluidics) [92, 93]. 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 [94, 95]. In 1997 Rashkovetsky et al. [96] 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 [97] and later by others [98][97][96][96][96]. 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 in 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 [97, 99]. 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.

6.3. Continuous Full Filling

Today, ESI is the dominating ionization technique used in liquid phase separations coupled with MS detection. As ESI originally was developed for HPLC, the performance of many of the earlier separations published on MEKC/MS were hampered due to the use of ESI interfaces not suited for the capillary format. As the ESI interfaces were miniaturized and adapted to the capillary format significant improvements in MEKC/MS were achieved. Further improvement was obtained with the orthogonal ESI-interfaces. These interfaces, spraying at an angle to the mass spectrometer inlet, had a greatly improved tolerance to non-volatile electrolyte constituents [100]. However, further developments are required to realize the full potential of CE/MS [71, 80, 101]. Viberg [24] elegantly showed that nanoparticles could be used as a PSP in a continuous full filling (CFF) technique using an orthogonal ESI interface (Figure 9) 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. Due to an orthogonal ESI interface the nanoparticles was exclude from entering the MS. 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 would risk entering the mass spectrometer. Several studies, mainly involving improvements in the nanoparticle based PSP, followed this proof of principle report [25, 102-108]. The later studies, including paper II, showed that CFF with nanoparticles could be performed without any negative effects on the analyte signal, without any contamination of the mass spectrometer and with efficiencies as high as one million plates per meter [25, 106, 107].
Figure 9. A schematic picture showing the principal of CFF-CEC/ESI-MS, where the added nanoparticles to the BGE are transported through the open capillary. This will allow for a separation of neutral analytes as well due to the analytes different partitioning 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 nanoparticles in EKC and related techniques has increased exponentially during the past two decades (Figure 10). Besides their ESI-MS compatibility, these phases also have beneficial mass transfer and a zero CMC. Despite developments of new PSP’s and 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 photo ionization (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 [80]. However, although proven to possess an extensive potential in EKC/MS, ESI-MS is generally still more sensitive [80, 109-111].

Figure 10. The number of published articles with a topic concerning nanoparticles and CE and the sum of PSP-CE related techniques (source ISI Web of knowledge).
7. Concluding remarks

"If you can’t explain it simply, you don’t understand it well enough"

Albert Einstein

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. 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 loosing sensitivity due to ion suppression. Despite the different ionization techniques in APPI and ESI the same spray needle is used. Therefore, several of the drawn conclusions could be used in paper II as well. Paper I indicated that optimizing the protruding capillary end from the stainless steel sprayer and that the nebulizing gas flow had a large influence on the detection sensitivity. 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 of optimize the conditions for CE-APPI/MS.

Based on paper I and II, and all the number of publications during the last decade, there are definitely a high interest in CE and CE-related techniques. But more research is needed to fully use their potentials. However, the use of MS friendly pseudostationary phases will lead to higher interest in the technique and hopefully become even more appreciated and used in important field such as clinical applications. The use of nanoparticles in sheathless ESI would be an excellent way of combining ESI friendly pseudostationary phases with the good properties of the sheathless interface, since paper II showed the importance of organic modifier in the BGE when working with nanoparticles in CE. When working with samples with high salt content, nanoparticles in CE-APPI/MS could however be the method of choice.
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John F. Kennedy

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9. References