Development of Capillary Electrophoresis Methods Coupled to Mass Spectrometry for Biomedical and Pharmaceutical Analysis

ANISA ELHAMILI
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Abstract

The analysis of large intact proteins and complex biological samples containing drug molecules is a common complicated task for many scientists. However, due to the importance of these molecules, there is a growing interest in pharmaceutical and medicinal research to develop rapid, highly sensitive and efficient analytical techniques. The advantages of capillary electrophoresis (CE) in combination with mass spectrometry (MS) provide a powerful analytical tool. However, further improvement and development of these techniques are required to extend their utility and to meet the challenges of selected analytes. Thus, the scope of this thesis deals with the development of novel analytical methods to achieve efficient and high performance analysis of peptides, intact proteins, digests of complex samples and basic pharmaceutical drug compounds in biological matrices.

Implementation of CE for routine analysis of proteins and complex samples is constrained by the partial adsorption to the capillary wall. Consequently, the use of surface modified capillaries is required to control the surface properties and prevent analyte adsorption. In this thesis, analyte adsorption was successfully prevented using tailored covalent cationic (M7C4I) and electrostatic cationic (PVPy-Me) coatings. Rapid and efficient separations of peptides, proteins and digests of complex samples such as cerebrospinal fluids were obtained with these coatings. The M7C4I coating showed a distinct ability to handle large intact proteins with a molecular size of over 0.5 MDa. The highest peak efficiencies and surprisingly high peak stacking effects were obtained by adding salts to the protein samples. The effect of salt additives on peak efficiencies of intact proteins was further demonstrated and compared using different surface modified capillaries. Additionally, rapid CE-ESI-MS quantification of pharmaceutical drug molecules in human plasma was performed after a SCX-SPE sample preparation method using the M7C4I coating. In conclusion, the results presented in this thesis show the strong potential of CE in combination with MS using electrospray ionization (ESI) for the analysis of peptides and large intact proteins and the applicability for clinical monitoring of the levels of pharmaceutical drug molecules in human plasma with high sensitivity and efficiency.

Keywords: Capillary Electrophoresis, Capillary Surface Modifications, Electrospray Ionization, Mass Spectrometry, Peptides, Intact Proteins, Basic Pharmaceutical Drug Molecules and Complex Biological Samples

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urn:nbn:se:uu:diva-143814 (http://urn.kb.se/resolve?urn=nbn:se:uu:diva-143814)
“They said, Exalted are you; we have no knowledge except what you have taught us”

_Holy Quran: 2: 32_

_To my parents  
To my sisters & brothers_
List of Papers

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

I  Rapid capillary electrophoresis time-of-flight mass spectrometry separations of peptides and proteins using a mono-quaternarized piperazine compound (M7C4I) for capillary coatings.  

II  Analysis of peptides using N-methylpolyvinylpyridinium as silica surface modifier for CE-ESI-MS.  

III  The effect of sample salt additives on capillary electrophoresis analysis of intact proteins using surface modified capillaries.  

IV  Optimizing the extraction, separation and quantification of tricyclic antidepressant drugs in human plasma with CE-ESI-TOF-MS using cationic coated capillaries.  

V  A method for quantitative analysis of an anticancer drug in human plasma with CE-ESI-TOF-MS.  
Anisa Elhamili, Jonas Bergquist. *Submitted to Electrophoresis*
Author’s Contributions

Paper I: I planned and carried out the CE-MS experiments in collaboration with Magnus Wetterhall. I performed all sample preparations and all the CE experiments and I wrote the paper.

Paper II: I planned the experiments with Magnus Wetterhall and carried out the CE-MS experiments together with Marcus Sjödin. I performed all sample preparations and all the CE experiments and I wrote the paper.

Paper III: I planned the experiments together with Magnus Wetterhall and Angel Puerta. I carried out all the experimental work and I wrote the paper.

Paper IV: I planned the experiments together with Magnus Wetterhall and Jörgen Samuelsson. I performed all the experimental work and I wrote the paper.

Paper V: I planned and carried out all the experimental work and I wrote the paper.

Papers not included in the thesis

- Improvement of coating stability and injection conditions for zone electrophoresis of proteins in capillaries coated with N, N-didodecyl-N, N-dimethylammonium bromide (DDAB).
  Anisa Elhamili, Douglas Westerlund, Jonas Bergquist, Stellan Hjertén. (Manuscript)

- A novel technique to study adsorption of proteins in coated and non-coated capillaries with special reference to capillaries coated with N, N-didodecyl-N, N-dimethyl ammonium bromide (DDAB).
  Anisa Elhamili, Douglas Westerlund, Jonas Bergquist, Stellan Hjertén. (Manuscript)
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### Abbreviations

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<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ACN</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>BGE</td>
<td>Background electrolyte</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CE</td>
<td>Capillary electrophoresis</td>
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<tr>
<td>CZE</td>
<td>Capillary zone electrophoresis</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
</tr>
<tr>
<td>Da</td>
<td>Dalton</td>
</tr>
<tr>
<td>EOF</td>
<td>Electroosmotic flow</td>
</tr>
<tr>
<td>ESI</td>
<td>Electrospray ionization</td>
</tr>
<tr>
<td>HV</td>
<td>High voltage</td>
</tr>
<tr>
<td>i.d.</td>
<td>Inner diameter</td>
</tr>
<tr>
<td>IT-MS</td>
<td>Ion trap mass spectrometry</td>
</tr>
<tr>
<td>LC</td>
<td>Liquid chromatography</td>
</tr>
<tr>
<td>LLE</td>
<td>Liquid-liquid extraction</td>
</tr>
<tr>
<td>MALDI</td>
<td>Matrix assisted laser desorption ionization</td>
</tr>
<tr>
<td>MEC</td>
<td>Minimum effective concentration</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>MS(^n)</td>
<td>Tandem mass spectrometry</td>
</tr>
<tr>
<td>MTC</td>
<td>Minimum toxic concentration</td>
</tr>
<tr>
<td>Mw</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>m/z</td>
<td>Mass over charge ratio</td>
</tr>
<tr>
<td>M7C4I</td>
<td>1-(4-iodobutyl) 4-aza-1-azoniabicyclo (2, 2, 2) octane iodide</td>
</tr>
<tr>
<td>o.d.</td>
<td>Outer diameter</td>
</tr>
<tr>
<td>pI</td>
<td>Isoelectric point</td>
</tr>
<tr>
<td>PVA</td>
<td>Polyvinyl alcohol</td>
</tr>
<tr>
<td>PVPy-Me</td>
<td>N-methylpolyvinyl-pyridinium</td>
</tr>
<tr>
<td>RSD</td>
<td>Relative standard deviation</td>
</tr>
<tr>
<td>SCX</td>
<td>Strong cation exchange</td>
</tr>
<tr>
<td>SPE</td>
<td>Solid phase extraction</td>
</tr>
<tr>
<td>TAC</td>
<td>N-trimethoxysilyl-propyl-N,N,N-trimethyl-ammonium chloride</td>
</tr>
<tr>
<td>TCA</td>
<td>Tricyclic antidepressant</td>
</tr>
<tr>
<td>TOF-MS</td>
<td>Time-of-flight mass spectrometry</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra-violet</td>
</tr>
</tbody>
</table>
1 Introduction

The analysis and detection of targeted components of interest is the best way to predict and understand changes in our surroundings, in our environment as well as in ourselves. These components could either be endogenous compounds such as peptides and proteins, or exogenous compounds such as pharmaceutical drug molecules. Peptides and proteins play a number of important biological functions as the building blocks of our cells and are key participants in many regulatory pathways. In addition, they can reveal biological conditions either in the disease state, diagnosis, prognosis and/or treatment strategies. Development of new methods for proteomic and peptidomic research is therefore of great importance in order to understand the main functions of the cells, for clinical diagnosis and for drug development. Pharmaceutical drug analysis is, on the other hand, highly important for correct and effective patient treatment, especially for drugs with a narrow therapeutic index. Moreover, drug analysis is connected with quality control of pharmaceutical products and biopharmaceutical development. Thus, there is a growing demand in many fields for the development of rapid, highly sensitive, reproducible and efficient analytical tools. Swedish scientists at Uppsala University have contributed with pioneering discoveries for proteins analysis. Theodor Svedberg developed the ultracentrifugation technique [1] and Arne Tiselius developed electrophoretic and adsorption chromatographic techniques [2]. They were both awarded the Nobel Prize for their work in chemistry, Svedberg in 1926 and Tiselius in 1948. One of Tiselius students, Stellan Hjertén, further developed the electrophoresis techniques in describing free zone electrophoresis using rotating quartz capillaries [3].

Capillary electrophoresis (CE) has been used in recent years as a separation tool for proteins and many other analytes [4]. The high throughput capacity of CE enables the analysis to be completed in a short time, compared to other separation technologies. Additionally, CE has the ability to handle minute sample volumes, which makes it an attractive choice in applications with limited sample volumes, such as the analysis of single cells, organelles and biofluids [5,6]. However, the use of CE for routine analysis of peptides, proteins and basic drug molecules has grown very slowly. This is due to the inherent properties of such analytes to adsorb either reversibly or irreversibly to the capillary silica wall, which in turn severely reduces the efficiency of the CE separation [7-10]. Thus, it is difficult to separate such analytes and/or cationic analytes in general without altering the chemistry at the silica wall.
Consequently, the development and use of suitable capillary modification is of prime importance to reduce analyte-wall interactions.

Mass spectrometry (MS) is a very powerful and informative analytical tool for detection and characterization of different molecules due to its high sensitivity, selectivity, accuracy and versatility. MS measures the mass-to-charge ratio of chemical species and gives information about the molecular weight and structure of the analytes. The combination of fast and high efficiency CE separations with highly informative MS detection, provides a high performing analytical system. CE-MS as a key tool in different research areas, particularly in pharmaceutical and biomedical fields, has grown very rapidly upon the introduction of different ionization techniques; particularly, and most importantly, electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI) [11]. The coupling of CE to ESI-MS was first done more than twenty years ago and since then this combination has grown continuously to become one of the most widely used analytical methodologies [12].

The aim of this thesis deals with development of CE methods and its online coupling and application in ESI-MS analysis of peptides, proteins and basic drug molecules. The analyte-wall interactions prevented by using either a covalent cationic or an electrostatic cationic coating for the separation of peptides, proteins and the digest of complex biological samples. It the first time such a covalent cationic coating has been used for CE analysis of very large proteins and provided extremely good peak shapes and efficiencies. In addition, it showed great potential for rapid, reproducible and high efficient CE-ESI-MS analysis of basic pharmaceutical drug molecules in complex biological matrices such as human plasma. Finally, aspects and strategies for CE analysis of extremely large intact proteins (up to 0.5 MDa) using different surface modified capillaries are discussed. The obtained results reveal the potential of CE-MS coupling for rapid and efficient analysis of peptides, large intact proteins and pharmaceutical drug molecules in biological matrices with high efficiency.
2 Capillary Electrophoresis (CE)

2.1 Fundamentals of CE

Electrophoresis is defined as the migration of electrically charged species induced by an electrical field in an electrolyte rich background [3,13]. Only charged species migrate in an electric field, neutral species are not affected. The positively and negatively charged ions are forced to the cathode and the anode, respectively. CE was first introduced in 1981 by Jorgenson and Lukacs [13-15] and since then the technique has rapidly developed into a versatile tool within the separation methods in analytical chemistry. The general applicability of the technique is reflected in diverse fields; for instance, pharmaceutical sciences [16-18], food analysis [19], forensic science [20], clinical diagnosis [21-23], biological laboratories and DNA studies [24-26].

CE is a miniaturized form of electrophoresis [14] where the separation is usually performed in buffer-filled capillaries made of fused silica. This material has excellent thermal, optical and electrical properties and an outer coating of polyimide makes the fused silica flexible and mechanically stable. The advantages of using narrow inner diameter capillaries were reported during the early 80s [13,14]. Nowadays, CE using fused silica capillaries with an inner diameter (i.d.) of 25-75 µm and an outer diameter (o.d.) of around 360 µm is a well-established analytical technique [13,27]. CE consists of a compilation of electrophoretic techniques and the most basic technique is known as capillary zone electrophoresis (CZE). Other methods are based on the same principles but that differ in the selection of electrolyte system including: capillary isoelectric focusing (CIEF) [28], which is used mainly for the separation of proteins in a pH gradient according to their isoelectric points, and capillary isotachophoresis (CITP) [29-31], which is often used for sample preconcentration. Another mode is capillary gel electrophoresis (CGE) [32], where the capillary is filled with an appropriate gel or polymer solution, allowing separation of analytes according to their size and shape. Additionally, to separate nonionic compounds, capillary electrochromatography (CEC) [33,34] is frequently used. In CEC, a stationary phase is either bound to the capillary wall [35-37] or packed in the capillary [38]. Alternatively, nonionic compounds can be separated by adding either liposomes [39,40], micelles [41] or micro emulsions [42] to the background electrolyte (BGE) to form a pseudostationary phase in liposome electrokinetic chromatography (LEKC), micellar electrokinetic chromatography
(MEKC), and micro emulsion electrokinetic chromatography (MEEKC), respectively. In this thesis, CZE (further in the text referred to as CE) was used for the separation and analysis of peptides, proteins, digest of complex samples and basic pharmaceutical drug molecules in biological matrices.

One important characteristic of CE is the simplicity of the instrumentation as schematically depicted in Figure 1. This consists of a fused silica capillary filled with buffer, two buffer reservoirs connected to a high voltage power supply, two electrodes and a detector coupled to a computer for data collection.

Figure 1. Schematic picture of CE instrumentation. The enlarged section shows the directions of the electrophoretic mobility and electroosmotic flow.

The CE separation can be performed simply by filling the fused silica capillary with an appropriate buffer solution and then injecting the sample into the capillary from the inlet side, which is the opposite side of the detector. The separation takes place via application of a potential difference across the capillary (usually 10-30 kV). The most commonly used detector is ultraviolet (UV) light, however, other detection techniques can be used. Mass spectrometry (MS) is one of the most universal and sensitive detector and due to its extreme sensitivity, speed and accuracy, has become a key tool for the detection and characterization of various molecules in combination with CE. In this thesis, CE was coupled on-line to MS using electrospray ionization (ESI) as the ion source.
2.2 Electrophoretic Migration and Electroosmosis

The CE separation is based on the difference in migration velocity of the charged analytes in the field depending on the charge-to-size ratio of the ions. The larger the ratio, the faster an ion migrates in the applied electric field. The velocity of an ion \( v_i \) is therefore proportional to the applied field strength [43,44] according to equation (1):

\[
v_i = \mu_i \times E
\]

where \( E \) is the applied electric field (a fraction of the applied voltage and the capillary length, \( \nu/cm \)) and \( \mu_i \) is the electrophoretic mobility of the ion. The electrophoretic mobility is affected by the electroosmotic mobility and the total or apparent mobility (\( \mu_{app} \)) for cationic compounds is subsequently a result of the sum of these two mobilities as given in equation (2). For anionic compounds, the difference between those two mobilities will determine the apparent mobility.

\[
\mu_{app} = \mu_{ep} + \mu_{eo}
\]

The electroosmotic mobility is directly proportional to the zeta potential (\( \zeta \)) and the dielectric constant (\( \varepsilon \)) of the BGE, and inversely proportional to the viscosity (\( \eta \)) of the medium. The electroosmotic mobility (\( \mu_{EOF} \)) can then be given by the Helmholtz-Smoluchowski equation (3):

\[
\mu_{EOF} = \frac{\varepsilon \times \zeta}{4\pi \eta}
\]

The electroosmotic flow (EOF) is the movement of liquid driven by the electrical field together with charges at the liquid surface. The EOF acts as a pumping mechanism that continuously pushes bulk solution and the analytes through the capillary towards the detector. It has a flat flow profile (compared to the parabolic flow in Liquid Chromatography LC) which contributes to retaining the high peak efficiencies. The origin of EOF is the negative charge on the inner wall of the capillary tube. The silanol groups (Si-OH, pK\textsubscript{a} \( \sim \) 3-9) on the capillary surface start to dissociate to anionic form (Si-O\textsuperscript{-}) when activated with a solvent above pH 3.0, giving a negatively charged surface. The cationic components of the BGE are electrostatically attracted to the negatively charged surface, leading to the formation of an electric double layer containing an excess of cations at the inner wall of the capillary [45-47], as depicted in Figure 2.
Figure 2. Schematic picture of the electric double layer structure. $\psi_x$ is the potential at the distance $x$ from the surface, $\psi_0$ is the surface potential, $\psi_\delta$ is the stern layer potential and $\zeta$ is zeta potential.

The first fixed layer near the silica wall is known as the stern layer, while the cations with their solvation shell in the second layer, the diffuse layer, will migrate toward the cathode in the presence of an applied voltage. Between the two layers a shear surface subsequently develops. The movement of cations will by friction forces rapidly spread throughout the BGE, which will move in the same direction with a velocity directly proportional to the applied field strength. The negative potential at the surface decreases rapidly in the stern layer to a value at the surface of shear called the zeta ($\zeta$) potential, generating an electroosmotic flow (EOF). The velocity of the EOF ($V_{EOF}$) can be defined by equation (4):

$$V_{EOF} = \frac{E \times \varepsilon \times \zeta}{\eta}$$

The zeta potential is determined by the surface charge on the capillary wall and thereby is strongly dependent on pH. Additionally, high ionic strength buffer leads to a reduction in the thickness of the electric double layer, which consequently reduces the EOF, due to the decrease of the zeta potential. The EOF is generally selected to have a direction from the injection to the detection side of the capillary and will thus ensure that all species present in the sample eventually pass the detector. In CE, the most charged
analyte with opposite charge to the electrode at the detector will elute first, with its electrophoretic mobility plus EOF mobility according to equation (2), whereas, analytes with the same charge as the detector electrode will elute later by the EOF mobility minus its mobility. The neutral analytes will elute in the time window between the negative and positively charged analytes; thus the neutral analytes cannot be separated by ordinary CE. The velocity of EOF can often be evaluated experimentally by using neutral and uncharged substances. Some requirements must be considered when selecting an EOF marker. The EOF marker should be uncharged over a wide pH range and soluble in the separation buffer. Moreover, it should be inert, easily detected and not interact with the capillary wall, which in turn enables the injection of small volumes. In this thesis, thiourea was used as EOF maker.

The efficiency of CE separation is generally much higher than other separation techniques and it is commonly referred to as a measurement of number of plate heights (H) and theoretical plates (N) as given by equation (5):

\[
N = f \cdot \left( \frac{t_i}{w} \right)^2 \quad (5)
\]

where \( t_i \) is the migration time, \( w \) is the width of the peak at half height or at baseline and the factor \( f \) is 5.54 when \( w \) is at half the height and 16 when the width of the peak at baseline is used. At high EOF, the zone will pass the detector at a high speed, resulting in a narrow peak with apparent plate number \( N_{app} \) or \( N_{ep+eo} \). Accordingly, the efficiencies corresponding only to the electrophoretic mobilities should be used for comparison of CE separation performance. Then the apparent plate number can be transformed to the true plate number \( N_{true} \) or \( N_{ep} \) by using equation (6):

\[
N_{ep} = \left[ \frac{\mu_{ep}}{\mu_{ep} + \mu_{eo}} \right]^2 N_{ep+eo} \quad (6)
\]

In this thesis, a number of theoretical plates were obtained by using the statistical moment method provided by the standard Agilent software, which gives a correct estimation of efficiency and compensates for peak asymmetry.
3 Modification of Capillary Silica Wall

The fused silica inner wall is naturally negative due to ionization of the free silanol groups at pH above 3.0, which then exist in deprotonated anionic form. The ionized silica wall thus shows a tendency to interact strongly with positively charged analytes. Such adsorption leads to fluctuation in the EOF and subsequently irreproducible migration times, severe band broadening, low recovery, decreased sensitivity and reduced separation efficiency [48-53]. This adsorption effect reduces the utility of CE as a routine analysis technique for peptides, proteins and cationic analytes in general. Therefore, modification of the inner wall of fused silica capillaries is highly beneficial for reducing analyte-wall interaction. Moreover, capillary wall modifications are advantageous for alteration of the EOF to achieve rapid separation, to increase resolution, reproducibility and to improve selectivity.

Over the years, different approaches to modify the capillary wall have been explored. In some instances, the adjustment of the pH of the running buffer to extreme pH values that give either a highly negatively or positively charged capillary wall were used [54-57]. For instance, the electrostatic interaction of proteins with the silica surfaces is affected by the pH. However, even if the net charge of proteins is the same as the net charge of the capillary surface, hydrophobic domains can still interact and cause problems [57,58]. In addition, the use of pH to control such adsorption effects has in many cases some limitations, such as protein aggregation [10]. Moreover, the adsorption effects of proteins to the capillary surface may depend on other factors such as the net charge of proteins, charge density and protein charge distribution. Other alternatives used to minimize analyte adsorption on the silica capillaries are high-ionic strength buffers [10,59] or buffer additives such as amines, low conductive zwitterions [60] or alkali metal salts [61,62]. However, for CE-MS, high amount of salts in BGE are not desirable as this causes loss of MS sensitivity. Additionally, high amount of salts causes Joule heating, due to the increase in current, leading to peak distortion. The more general approach to prevent analyte adsorption is to use surface coated capillaries [45,52,63-65]. Capillary coatings can be performed either by covalent coupling to the silanol groups on the capillary silica surface or by non-covalent modification using adsorbed polymeric coatings. Capillary coatings, especially the covalent ones, are the most preferred strategies when MS is used as a detection technique, since the other strategies are often not compatible with the MS detection due to the ion suppres-
sion effect and high background signals which decrease the sensitivity [66,67]. There are several requirements that have to be considered when evaluating capillary coatings for CE [48,68]:

- The coating layer must be homogenous to give full coverage of the silica surface and effectively shield the silanols group.
- The coating should maintain reproducibility of migration time, be easy to generate, maintain a stable EOF and be applicable for a wide range of buffer conditions.
- The coating should reduce peak tailing and give high recovery and high separation efficiency (typically around one million plates/m).
- The coating should be highly stable and compatible with the applied detection techniques.

3.1 Covalent Cationic Coatings

Covalent coupling to the ionized silanol group can be achieved either by using neutral or charged coatings. Covalent coatings with a variety of neutral and hydrophilic reagents are used mainly with long-term stability to mask the anionic sites on the capillary wall [45,52]. The most commonly used are polyacrylamide (PAA) [6,69-71] and polyvinlyalcohol (PVA) [72-74]. Neutral coatings have no restrictions regarding the charge of the analytes and efficient separation of both acidic and basic proteins using such coatings has been demonstrated [72]. However, the non-ionic nature of the neutral coating eliminates the EOF. The migration of the analytes therefore depends only on the electrophoretic mobility, which in turn prevents detection of both acidic and basic proteins in the same run. Other limitations include instability of the siloxane bond (Si-O-C) at neutral and alkaline pH and the coating process (silanization) is often time-consuming, involving multi-step processes which may introduce problems with irreproducibility. The major concern is the difficulty to regenerate the coatings, especially when dealing with complex biological samples. Although most of the permanent neural coatings reported so far can easily be used in CE-MS applications, due to their stability [75], such coatings do not induce an EOF, which is disadvantageous for the electrospray stability, especially when using sheathless interface. Such problems can be circumvented by using charged coatings (positive or negative) covalently attached to the silica wall, which generally give high EOF [48]. For cationic coatings, the pH of the BGE should generally be low, whereas for anionic coatings high pH is recommended.
It is generally more straightforward to carry out deactivation of the fused-silica surface with covalent cationic coatings. A stable and high EOF can be obtained which fulfils the requirements for ESI-MS detection. Moreover, with many covalent cationic coatings, the capillary can rapidly be regenerated within a few minutes by rinsing it with the coating solution [58,76]. During the 1990s, covalently bound 3-(aminopropyl)trimethoxysilane (APS) was the prominent coating reported [77]. It gives high reversed EOF and was used for CE-MS analysis of peptides and proteins using a sheathless interface [78]. Novel ω-iodoalkylammonium salts have been synthesized by Sebastiano and co-workers and have shown to be effective agents for permanent derivatization of fused silica capillaries [65,79,80]. One of these salts is the monoquaternarized piperazine compound [1-(4-iodobutyl) 4-aza-1-azoniabicyclo (2,2,2) octane iodide] (M7C4I). The M7C4I piperazine compound interacts with the ionized silanol groups via three different interaction mechanisms at alkaline pH: the predominant one is a covalent bond (alkylation) with the iodobutyl chain. The others are hydrogen bond formation via the tertiary amino group and an ionic bond with the neighbouring dissociated silanols groups via the quaternary amino group (Figure 3).

Figure 3. Reaction of monoquaternarized piperazine with the silanol group on the capillary wall (picture modified from [65,81]).

Thus, the M7C4I imparts a positive charge to the wall, reversing the charge of the surface and producing a high and stable EOF [65,79,80,82]. The M7C4I compound acts as a tri-functional derivative (or mixed mode) and is able to bind in a completely different fashion from that of conventional amino coatings. Based on this tri-functional binding mechanism, M7C4I has
been nicknamed (Scorpio) \[65,79\], since its structure and behaviour resemble that of a Scorpion, possessing two “chelae” and a “stinging” tail, as depicted in Figure 3. The M7C4I coating has been used successful for CE-UV separations of peptides, proteins and small organic molecules \[79-83\]. Covalent cationic coatings (M7C4I) were used in Papers I, III, IV and V to reduce analyte-wall interaction. In Paper I, the M7C4I coating was investigated for the first time as a surface derivatization reagent for on-line coupling of CE to ESI-MS, for fast analysis of a mixture of neuropeptides, intact proteins and digests of complex biological samples. The capillary coating process is very simple and fast within two minutes and the surface coating showed good stability over a wide pH range. Additionally, the M7C4I surface gave good repeatability in the migration times for repeated separations of peptides and proteins without having a recoating step in between the runs. Owing to the simplicity of the coating procedure, the capillary can rapidly be regenerated after a series of runs, if necessary. The obtained surface yielded rapid CE-ESI-MS separations of a mixture of neuropeptides and proteins within five minutes with high efficiencies (Figure 4).

There was no observed bleeding of the coating reagent into the mass spectrometer and no regeneration of the surface was needed between the runs. This is important particularly for the analysis of complex samples in order to prevent the potential of memory effects. The coating also showed the ability to handle large intact proteins (669 kDa) with retained peak shape and effi-

![Figure 4. CE-ESI-TOF-MS separation of A) peptides and B) proteins on M7C4I coated capillary using sheath flow interface ESI-MS as described in Paper I.](image-url)
ciency as will be discussed in chapter 6. In Paper III, the usefulness of a M7C4I surface was further evaluated for the analysis of large intact proteins in comparison to other capillary coatings. In Papers IV and V, the M7C4I was also investigated and used as a valuable surface for the pharmaceutical analysis of basic drug molecules as demonstrated by analysis of tricyclic antidepressant (TCA), beta-blocker and the anticancer drugs (Imatinib).

3.2 Electrostatic Cationic Coatings

Non-covalent modifications are usually prepared by flushing the capillary with the adsorbed coating reagent or by adding a certain amount to the BGE to continuously coat the capillary wall (dynamic coating), thus reducing analyte adsorption and modifying the EOF velocity [18,45,47,63,68,84,85]. Different types of amines, surfactants and polymers have been applied, which are reversibly bound to the negatively charged silica wall. Based on the type of coating used, the negative charge of a silica surface is reduced, neutralized or even reversed [86]. Over the years, a wide variety of non-covalent coatings have been reported that have been used successfully for CE separations including for instance; polybrene [5,58], polyethylenimine [58,87,88], poly(methoxyethoxyethyl)ethyleneimine [58], poly(diallyldimethylammonium chloride) [58,76], Cetyltrimethylammonium bromide [85] and polyarginine [89]. In addition, Poly-E323 [90,91] and Poly-LA 313 [92] was synthesized by our group and the new copolymer ethylpyrrolidine methacrylate (EPyM)/methyl methacrylate (MMA) was synthesized by Cifuentes and co-workers [93]. These coatings are mainly characterized by their simplicity of use and ease of optimization. However, from a CE-MS perspective, the presence of additives in the BGE, or bleeding of some adsorbed polymers, contaminate the ion source and cause ion suppression which in turn decrease the sensitivity [8,94] as discussed previously. Thus, such properties can limit their use for CE-ESI-MS.

Electrostatic cationic coatings are usually made by flushing the capillary with the coating solutions, generating a positive surface with reversed EOF. The coating materials are usually polymers with a large number of cationogenic amine groups, to facilitate electrostatic interactions with the silanols group [68]. Enhanced adsorption of such polymers is controlled generally by an increase in the silica surface charge density by initial rinsing steps using sodium hydroxide [49]. A novel cationic polymer coating consisting of an N-methylpolyvinylpyridinium quaternary ion (PVPy-Me) (Figure 5) was synthesized and reported by Sebastiano et al. [95].
Figure 5. Chemical formula of N-methylpolyvinylpyridinium coating.

The PVPy-Me surface interacts electrostatically with the ionized silanol groups, giving a cationic surface with a reversed EOF which thereby reduces the adsorption of cationic analytes to the silica wall. The PVPy-Me polymer was used for CE-UV analysis of peptides and small proteins [95]. In Paper II, the potential and compatibility of PVPy-Me polymer as a silica surface modifier for CE-ESI-MS analysis of peptides, proteins and complex biological samples was investigated for the first time. The capillary coating process is simple and no reconditioning of the capillary surface with the coating solution was needed between the runs. At acidic pH, the surface modifier gave fast and repeatable separations of peptides, proteins and protein digests for more than four hours of continuous use. The coating also showed the ability to handle intact proteins with a maximum Mw of 80 kDa with retained peak shape and peak efficiency. The surface coating is compatible with ESI-MS application and facilitates rapid analysis of complex peptide mixtures with a high sequence coverage and identification score. One important characteristic of the PVPy-Me polymer is the decrease in the EOF mobility at pH above 7 to a value equal to zero (Figure 6). This is an interesting observation which could be useful in some applications since it is rather uncommon to obtain charged physically adsorbed polymers which have such a property. However, it is disadvantageous if a sheathless interface is used, as stable and high EOF is required.
Figure 6. Mobility of EOF as a function of pH of the running buffer on a PVPy-Me coated capillary and a bare fused silica capillary, as presented in Paper II.
Mass spectrometry (MS) has become a key tool and the analytical technique of choice in many application areas for the detection and characterization of various molecules due to its high sensitivity, selectivity and accuracy [8,96]. MS separates charged ions, based on their mass-to-charge ratio ($m/z$) in gas phase, by applying an electric or magnetic field and measures their relative abundance. It also gives information on the molecular weight of the analytes, structure and enables the separation of co-migrating molecules. Although different MS instruments available today may have different properties and different operation modes, in principle they all consist of the same major building blocks: an ion source, ion optics, a mass analyzer, a vacuum pump, a detector and a data handling system, as schematically shown in Figure 7.

Figure 7. Schematic picture of the general components of an MS instrument with reflectron time-of-flight mass analyzer.
4.1 Coupling of CE to MS

Although MS has good capacity to analyze many samples, including complex biological samples, the best sensitivity and selectivity is obtained by implementing a separation step prior to the MS analysis. The combination of a rapid and efficient CE separation technique with highly sensitive MS detection provides a powerful analytical tool and further extends the utility of CE. As the sample capacity in CE is low, the high sensitivity of MS makes it possible to detect low abundant species. The first attempt to combine the two techniques began in the mid 1980s and the first publication describing this hyphenation was in 1987 by Smith and co-workers [12]. Today, CE-MS has increasingly been used for many analytical purposes and is particularly suited for the analysis of highly polar and charged analytes which could be difficult to handle using LC-MS methods [97]. CE-MS has been applied, for instance, in proteomics [66,98-101], metabolomics [102-104], food analysis [105], forensic science [106], pharmaceutical analysis [107-112] as well as for the analysis of intact proteins and their isoforms [16,97,113-115].

Analysis and detection of an analyte using MS detection requires the molecules to be ionized and in gas phase. This could be achieved by heating the sample into gas phase, but some thermo labile analyte, such as proteins, may be affected adversely by heating. To overcome this problem, many ion sources have been developed to achieve this transition between phases. The most commonly used ionization technique for liquid samples is electrospray ionization (ESI) [116]. Apart from ESI, other ionization techniques performed at atmospheric pressure (API) can be coupled to CE, such as atmospheric pressure chemical ionization (APCI) and atmospheric pressure photo ionization (APPI) [117,118]. Matrix assisted laser desorption ionization (MALDI) is another popular ionization technique typically performed in a vacuum inside the mass spectrometer [119], which considerably limits the practical online interfacing of CE with MALDI-MS. However, off-line coupling can be used with independent optimization of the CE and MS conditions. The most commonly used strategy for off-line coupling is by fraction collection into nano vials, after which the sample is deposited onto a MALDI target plat [120]. Alternatively, the eluent can be collected directly onto the target either continuously or stepwise. ESI-MS and MALDI-MS are commonly used ionization techniques for analysis of biomolecules [11].

4.1.1 Electrospray Ionization (ESI)

The process of electrospray has been known for at least two centuries and was first proposed as an ion source for MS by Dole et al. [121]. The potential of ESI for MS analysis was further demonstrated by Fenn and co-workers [122-124]. Since then, ESI has become one of the most frequently used ionization techniques due its ease of use, versatility and effectiveness
for charging large biomolecules [48]. ESI is the process of transferring solutes from liquid to gas phase and adding charge to the analytes [125,126]. These are important features since many separation techniques used today such as LC and CE are conducted in solution. One main feature of ESI is the formation of multiply charged ions of large molecules without fragmentation (soft ionization) and the lowering of $m/z$ values to a range easily measured by different types of mass analyzers [127]. However, multiple charging yields a more complex mass spectrum to interpret since the analyte signal spreads out on several peaks, which could also reduce the sensitivity. To achieve this ionization process, a strong electric field (3-6 kV) under atmospheric pressure is applied between the liquid delivering capillary outlet and the inlet of the MS. The process gives rise to charged droplets at the electrospray tip and the solvent in these droplets is then evaporated and highly charged ions with high analyte concentration are produced [116,128,129]. The repulsion of the positive ions and the attraction of the electric field on the positive ions overcome the surface tension of the liquid and expand the liquid into a Taylor cone. These ions are then transferred to the MS for separation based on their $m/z$ ratio, followed by MS detection (Figure 8).

![Figure 8. Electrospray ionization in positive ion mode where the ions are generated and transferred into gas phase.](image)

When interfacing CE to MS using ESI as an ion source, several important requirements have to be considered and carefully optimized. One important parameter is the pH of BGE, since both separation and MS detection will be affected by the pH. The charge state of the proteins for instance is strongly dependent on pH. The use of an acidic buffer (pH<$pI$) results in an increase of protein protonation, a decrease in protein $m/z$ ratio and contributes to the
production of multiply charged species. Generally, the use of BGE at low pH is common in CE-MS due to its compatibility with MS detection and it is favored, especially with the positive ESI-MS mode [48]. In contrast, the use of high pH BGE is less frequently encountered due to problems with analyte stability and solubility. The use of organic solvents is another factor that needs considering to enhance electrospray formation and its stability. However, in some cases this can cause protein denaturation. Capillary coating stability is also required and analyte-wall interaction should be minimized [8,94] as discussed in chapter 3. Moreover, a relatively high and stable EOF is often needed to enhance the ESI stability, especially when using a sheathless interface [48,130]. Stable and successful CE-ESI-MS depends also on careful optimization of instrumental parameters such as the effluent liquid (flow rate) and the source itself (geometry and applied voltage) [131].

An additional key requirement for efficient CE-ESI-MS coupling is to consider two electrical circuits, one running the CE and one feeding the ESI [66]. In order to provide two closed circuits, the electric contact must be made with the CE column outlet. Over the past twenty years, a wide variety of interfaces have been proposed to meet these requirements and to maximize the potential of CE-ESI-MS hyphenation and these have been summarized in a number of reviews [94,100,108,116,132-136]. These include either the addition of liquid, the co-axial sheath flow interface [137] and the liquid junction interface [138,139] or without the addition of liquid, the sheathless interface [140-142]. In Papers I, II, IV and V, the co-axial sheath flow interface was used for the on-line coupling of CE to ESI-MS.

From the early year of CE-MS applications, the co-axial sheath flow interface is the most popular CE-MS interface due to its robustness and ease of implementation [143,144]. The co-axial sheath flow interface consists of a central CE capillary surrounded by a stainless steel electrospray needle and nebulizer gas. A volatile sheath liquid is introduced in the space between the ESI needle and the CE capillary; this maintains the electric contact and facilitates the ESI process (Figure 9).
The co-axial sheath flow interface allows independent optimization of the separation buffer, and in addition, it is beneficial when organic modifiers cannot be added to the separation buffer. Moreover, because the electrospray formation is not dependent on the flow of BGE from the separation capillary, the co-axial sheath flow interface enables coupling of CE to ESI-MS in an application where EOF is low or eliminated [145]. The sheath liquid also serves to increase the liquid flow to levels comparable to those in LC. However, the major drawback of this interface is the risk of dilution and back migration of ions from the sheath liquid to the separation capillary, which in turn affects the pH and separation performance. Nevertheless, the dilution of CE effluent is inherent to the sheath liquid interface, which could reduce sensitivity. It is worth mentioning that it might not be significant, since the sheath liquid is also evaporated during the spray process [144]. Careful optimization of the sheath-liquid composition, the sheath-liquid flow rate and the position of the capillary tip will greatly improve the limit of detection (LOD) and the ESI stability [45].

4.2 Mass Analyzers

Almost all mass analyzers, such as quadrupole, ion trap (IT), time-of-flight (TOF) and Fourier transform ion cyclotron resonance (FTICR) can be coupled on-line with CE, generally by using ESI as the ionization source [144]. Because increased resolution and high accurate determination of molecular masses are often required, TOF-MS can be advantageous due to its unlimited mass range, high speed, sensitivity and its potential for high duty cycle. Additionally, TOF-MS has gained wide acceptance in the analysis of macromolecules and is considered the analyzer of choice for CE-MS applications.
IT-MS is on the other hand allows multistage MS analysis (MS^n) and provides additional information by means of the multistage fragmentation of the analyte [147]. Cifuentes and co-workers have recently compared the performance of CE-TOF-MS and CE-IT-MS in terms of LOD, sensitivity, selectivity and repeatability in the determination of relative molecular mass [148,149]. In this thesis, TOF-MS and IT-MS have been coupled on-line to CE using ESI and sheath flow interface.

4.2.1 Time-of-Flight Mass Spectrometry (TOF-MS)

TOF-MS was first introduced in 1946 by Stephens. However, CE-TOF-MS coupling was only investigated in the 1990s [150,151]. The principle of TOF-MS is based on the movement of ions in the same direction having a constant kinetic energy but different masses. Ions will have a corresponding distribution of velocities inversely proportional to the square root of m/z. Thus, the arrival times of the ions will be distributed according to the m/z ratio [144]. Low m/z ions fly faster than high m/z ions and are thus separated from each other. In MS, the resolving power is mainly measured by the ratio of \( m/\Delta m \), where \( \Delta m \) is a discernable mass difference. In TOF-MS, the resolving power is measured in terms of \( t/\Delta t \), where \( \Delta t \) is the width at half the height of the peak. The resolving power is therefore limited by small difference in the measured flight times of ions of the same mass [144]. The high mass accuracy, high mass resolution, theoretically unlimited m/z range and relatively low cost are prominent features of TOF-MS [113,152]. Also, TOF-MS has a high data acquisition rate and produces a sufficient number of data points across a peak [113,130]. This meets with the CE requirements of very small peak widths produced by the fast and efficient CE separations, which makes TOF-MS the analyzer of choice for CE [146]. ESI and MALDI are commonly used ion sources for CE-TOF-MS coupling [67,153].

The first TOF mass spectrometer was of fairly simple construction and known as linear TOF. Ion distributions degraded mass resolution in TOF-MS [144]. The development of two technologies, reflectron (ion mirror) and orthogonal acceleration (oa), has contributed to the increased interest in TOF-MS [152]. The reflectron creates a retarding field to correct for the initial position and velocity dispersions in TOF-MS. Thus, the high and low energy ions are focused at the detector plane and improve the resolution [144,152]. The oa was fully described in the late of 1980s [154] in which the ionization source, the accelerator and the detector express an angle slightly greater than 90°. It is characterized by high efficiency (duty factor) in gating ions from an external continuous ion source, by simultaneous correction for velocity and spatial dispersions and capability of mass resolving powers ten times higher than a conventional TOF-MS instrument [144,152,155].
In **Paper I**, the CE-ESI-TOF-MS was used for separation of a mixture of neuropeptides, proteins and tryptic digests of bovine serum albumin (BSA) and cerebrospinal fluid (CSF). The analysis was performed in less than five minutes with high efficiency and high sequence coverage (Figure 10).

![Figure 10](image)

**Figure 10.** CE-ESI-TOF-MS analysis of tryptic digest of A) BSA and B) CSF on a M7C4I coated capillary using sheath flow interface, as described in **Paper I**.

In **Papers IV** and V, the CE-ESI-TOF-MS was used for the analysis and quantification of basic pharmaceutical drug molecules in biological matrices, TCA drugs and an anticancer drug (Imatinib) respectively, after appropriate sample pre-treatment conditions. In **Paper IV**, all the TCA analytes tested (imipramine and clomipramine) and their major metabolites (desipramine and norclomipramine) could readily be detected with TOF-MS and the CE-MS separation of the TCA analytes was conducted in less than ten minutes with good peak shape and high peak efficiency (Figure 11). The same performance was obtained for the CE-ESI-TOF-MS analysis of an anticancer drug (Imatinib) as presented in **Paper V**.
Figure 11. Extracted ion chromatograms of the CE-ESI-TOF-MS analysis of clomipramine (m/z 315.1) and norclomipramine (m/z 301.1) as presented in Paper IV.

4.2.2 Ion-Trap Mass Spectrometry (IT-MS)

Ion trap was first invented in 1953 by Paul et al. [156,157] who was awarded a Nobel Prize in 1989 for his work. An interesting review covering the historical aspects of IT-MS was published in 1999 by Todd et al. [158]. Nowadays, IT-MS has been used for different applications such as analysis of peptide and protein [159], analysis of carbohydrates [160] and organic compounds [161]. IT-MS consists of an arrangement of three electrodes, a hyperbolically shaped ring electrode and two hyperbolically shaped end cap electrodes. Trapping or storage of externally produced ions is achieved by applying a high voltage called radio frequency to the ring electrode while the end cap electrodes are kept grounded. Simultaneous trapping of the ion can be obtained by changing the direction of the field every time the ion is approaching the electrodes. An interesting review focused on the general principles of IT-MS was published by McLuckey et al. in 1994 [162].

Tandem mass spectrometry (MS^n) in the IT entails mass isolation to eject all ions except the ion of interest (precursor ion). Thus, only product or fragment ions related to the precursor ions are present in the fragmentation step of a particular m/z value by collision with helium atom within the IT. The process of isolation, fragmentation and mass analysis can be repeated several times and is known as MS^n [163]. In Paper II, MS/MS was used for the analysis and identification of the tryptic digest of complex samples.
5 Sample Preparation

In any chemical analysis, the analytical method must include the following steps: sampling, sample preparation, separation, detection, characterization and data evaluation (Figure 12). Sample preparation is therefore an integral and essential part of the chemical analysis and it is considered as bottleneck in the entire analytical process, since it is often causes the largest variability in the analytical results. Consequently, the quality of sample preparation is a vital factor in determining the success of the analysis and accordingly careful optimization of the sample preparation procedure is necessary [164,165].

Figure 12. The different steps involved in any chemical analysis.

The key functions of any sample preparation technique are either clean-up, extraction or pre-concentration of the analytes to improve the quality of the analytical results. Typically, there is no general sample preparation method available since any sample preparation method is dependent on a number of factors, mainly the nature of the analyte, the intended purpose and the subsequent applied analytical technique. In addition, several sample treatment steps are sometimes required to get the correct sample analysis whereas a single treatment step could be enough in other cases. Thus, selection and optimization of an appropriate sample preparation method is not straightforward, particularly and most importantly when dealing with highly complex samples such as biological matrices (tissue, whole blood, plasma, serum, urine, etc). Preferably, sample preparation should be performed with the minimum loss of analytes while eliminating as many interfering substances as possible. Moreover, it should be a rapid and simple process in order to reduce the time required and subsequently to reduce number of steps
which can often introduce errors and can lead to a loss of analytes [164,165]. Currently, CE is one of the most widely used analytical tools due to its advantages for analyzing a vast variety of analytes [66]. However, a disadvantage is the low sensitivity of CE, due to the small dimension of CE capillaries that can handle only small sample volumes, which in turn necessitates using a suitable sample treatment process and also a sensitive detector such as MS. Moreover, despite the high selectivity and sensitivity of the MS, direct injection of crude complex samples could suppress the ionization or even cause a complete loss of the MS signal as discussed earlier. Thus, different sample treatment strategies have been proposed prior to CE-MS analysis to reduce sample complexity and to improve LOD include: liquid-liquid extraction (LLE), solid-phase extraction (SPE), protein precipitation and ultrafiltration [112,165,166]. In Papers I and II, tryptic digest and desalting of complex biological samples was conducted using ZipTip C$_{18}$ columns. In Papers IV and Paper V, the extraction of basic pharmaceutical drug molecules from human plasma was performed using selective strong cation exchange (SCX) SPE columns and LLE technique.

5.1 Liquid-Liquid Extraction (LLE)

Liquid-liquid extraction, also known as solvent extraction and partitioning, is the classical sample preparation method. It separates analytes based on their relative solubilities into two different immiscible solvents. Thus, the selectivity and efficiency of such an extraction process depends greatly on the proper selection of the immiscible liquids. In addition, other factors such as the pH and the addition of salts and complexation agents can affect the distribution of the analyte in both phases. Different studies have shown that using LLE for drug molecules in biological matrices and other endogenous components results in good extraction efficiency and clean up [164,165]. In Paper V, LLE was used for the extraction of an anticancer drug (Imatinib) from human plasma.

5.2 Solid-Phase Extraction (SPE)

Sample preparation using SPE was first introduced in the mid 1970s, replacing LLE. Since then, SPE has gained wide acceptance due to its simplicity, selectivity, high extraction recovery, reproducibility and improved LOD [164,165]. The use of on-line or off-line SPE is by far the most widely and commonly used sample pretreatment procedure prior to CE-MS analysis. SPE columns with different chemistries, adsorbents and sizes are available commercially, thus there is a wide variety to select from depending on the analyte and application.
5.2.1 Reversed Phase SPE

Reversed phase SPE columns extract analytes based on their polarity. The SPE media is made up of a hydrocarbon chain (C-4, C-8 or C-18) which retains analytes of mild to low polarity. The analytes are eluted by non-polar solvents to disrupt the interaction between the analyte and SPE media. In Papers I and II, the tryptic digestion of biological samples, CSF and BSA, were conducted, as described by Bergquist et al. [167] and the resulted digests were desalted using ZipTip C18 columns with a binding capacity of approximately 1 µg. After desalting and before CE-MS analysis, the peptides were reconstituted in 50% ACN, which renders in a peptide concentration of about 0.3 µg/µL (Figure 13).

![Figure 13. CE-UV analysis of tryptic digests of BSA on PVPy-Me coated capillaries desalted using ZipTip C18 columns, as presented in Paper II.](image)

5.2.2 Ion Exchange SPE

Ion exchange columns extract analytes based on electrostatic interaction of a charged group on the analytes and an opposite charged group on the SPE media. In this case, the pH of both buffer and sample should be selected to have both the analytes and the SPE media charged. Various kinds of ion exchange SPE have been described, including strong cation exchangers (SCX) and strong anion exchangers (SAX), weak cation exchanges (WCX) and weak anion exchangers (WAX). The analytes of interest can be eluted with a solvent that neutralizes the interaction. In paper IV, SCX-SPE columns were used to extract TCA drugs and their major metabolites from complex biological fluids (human plasma). The SCX-SPE columns used in paper IV are specifically suited for the selective extraction of basic drug
molecules from complex biofluids. The principle is based on a restricted access of proteins at the media surface and a core functionalized with the SCX group. All the TCA analytes tested have high pKₐ values and hence they are positively charged at acidic pH. Thus, the TCA analytes can access the media and be retained by their charge interaction, whereas proteins and other interfering substances are excluded. Upon washing with a solution of high pH, the analyte of interest can be eluted. A high and reproducible extraction recovery was achieved, compared to using either non-modified or modified spin filters with a hydrophilic polymer (Figure 14).

![Figure 14](image)

Figure 14. The extraction recoveries for the TCA drugs at the MTC with modified and non-modified spin filters and SCX-SPE columns using CE-ESI-TOF-MS, as presented in Paper IV.

The extraction recoveries ranged between 87-91% for TCA standards and also for TCA analytes extracted from spiked human plasma. These values were consistent at the minimum effective concentration (MEC) and minimum toxic concentration (MTC) with % RSD value of 1.7%. In addition, very clean extract and hence clean MS spectra were obtained using the SCX-SPE column and no peaks from the matrices, other than the analyte of interest, could be detected. Moreover, stable spray was obtained throughout the ESI-MS experiments, in contrast of using either non-modified or modified spin filters. When analyzing complex biological samples such as human plasma, this is a very important feature, which will not reduce the MS sensitivity and therefore facilitates the subsequent identification and quantification with high quality. In Paper V, the SCX-SPE column was further investigated for selective extraction and quantification of an anticancer drug (Imatinib) from human plasma in comparison with LLE. Consistent results and a high extraction recovery up to 91% were obtained using the SCX-SPE column, as in paper IV.
6 Protein Analysis

6.1 CE Analysis of Large Intact Proteins
The analysis of large intact proteins by CE is a demanding task, especially compared to the analysis of peptides and small proteins (< 20 kDa). This is mainly due to protein adsorption to the silica wall via different interaction mechanisms; electrostatic, hydrophobic and hydrogen bonding which strongly increase with increasing the molecular weight (Mw) [57,58,92] as previously discussed in chapter 3. Over the years, different efforts have been made to analyze protein samples using CE. Excellent reviews covering the progress in CE analysis of protein have been given by Dolnik and co-workers 1997-2005 [4,168-172] and by El Rassi et al. 2007-2009 [173].

In Paper I, the M7C4I coating showed the ability to handle large intact proteins up to 669 kDa with retained peak shapes and good peak efficiency. The best analysis condition for such large proteins was achieved at acidic pH, where both the capillary wall and the proteins have a net positive charge and thus repel each other. Surprisingly, Paper I is the first report of CE analysis of extremely large proteins with good peak shape and efficiency. Previous studies by Pontoglio et al. [81] indicate that the separation of proteins larger than 30-40 kDa can quickly deteriorate, although the same coating and similar pH of the BGE were used. Unexpectedly, the best performance for such large proteins regarding peak shape and efficiency was obtained by adding sodium chloride (NaCl) salt to the protein samples. Such findings had not been discussed or predicted by Pontoglio et al. [81]. Accordingly, extensive studies concerning the effect of sample salt additives on the analysis of intact proteins were conducted in Paper III.

6.2 Effect of Sample Salt Additives
Generally, the use of high ionic strength buffers or samples will cause the CE separation to deteriorate, cause band broadening and poor separation efficiency due to the development of Joule heating [174]. However, a surprisingly positive influence of adding salts to the protein sample regarding the CE peak efficiency was observed in Paper I. Comparable positive effects have previously been reported by Shihabi et al. [175-179] when both acetonitrile (ACN) and salts were added to the sample. In those studies, ad-
dition of a mix of ACN and salt to the sample generates a larger stacking effect than adding salt alone. However, it was not applicable to protein analysis as proteins are prone to precipitate in a high concentration of ACN. Additionally, the conductivity in the sample plug was lower than for the BGE and the stacking effects occurred due to the differences in conductivity. Thus, in Paper III, the effect of adding salts alone, at different concentrations to the protein sample of different sizes ranging from 14 kDa up to 669 kDa was investigated. The salt concentration in the sample varied between 0-100 mM while the ionic strength in the BGE was kept constant and no organic modifiers were added to the sample. Furthermore, other coatings yielding positively charged or neutral surfaces were evaluated and compared with the M7C4I coating. Although the conductivity in the sample plug was in most cases higher than that in the BGE, a remarkable peak stacking effect could still be observed (Figure 15).

![Figure 15](image_url)

Figure 15. CE-UV analysis of γ-globulin (Mw: 165 kDa) on M7C4I coated capillary as a function of NaCl concentration (0-100 mM, increase by 10 mM for each recording starting from the bottom) as described in Paper III.

The obtained results indicated that increasing the salt concentration in the protein samples gave a sharpening of the protein peaks with very high efficiency of up to two millions plates/m. Interestingly, there was a difference in the optimal concentration of salt needed for each protein to yield the highest peak efficiency. This could be related to the difference in the degree of hydrophobicity, the molecular size, the charge and the structure of the different proteins. A possible explanation for the improvement in peak efficiency could be a pseudo isotachophoretic phenomenon caused by the added salts, as previously reported by Shihabi et al. [179]. Other possible explanations
could also be increased protein charging by the added salt. High Mw proteins have the potential to bind a higher amount of salt ions than low Mw proteins. The hypothesis of protein charging effect was further evaluated using different molar concentrations of various Mw proteins. The obtained results showed that increasing protein molar concentrations necessitates increasing the salt concentration to the sample to achieve the highest peak efficiency, and this was consistent with all the tested proteins (Figure 16).

![Figure 16](image.png)

Figure 16. Peak efficiencies for different molar concentrations of \(\gamma\)-globulin as a function of salt concentration using M7C4I coated capillaries, as presented in Paper III.

Thus, the optimum molar concentrations of salt required in the protein sample to reach optimal peak efficiency appear to be dependent on both the Mw and the molar concentration of the protein. Other coatings besides the M7C4I coating were evaluated and compared; PolyE-323, TAC and polyvinyl alcohol (PVA) surface. By adding salts to the protein sample, significant improvements in peak shape and efficiency were observed for all except Poly E-323 (Figure 17).
Figure 17. CE-UV analysis of transferrin (80 kDa) on A) M7C4I, B) TAC, C) PolyE-323 and D) PVA coating. Positive polarity was used for PVA coating while negative polarity was used for the other coatings, as presented in Paper III.

The PolyE-323 coating interacts electrostatically with the ionized silanol groups [90,91] and the presence of salt is likely to reduce such interactions between the polymer and the silica wall. The TAC coating on the other hand is a covalent cationic monomer [180] and the highest peak efficiencies were obtained at a lower salt concentration compared to the M7C4I coating. This can be related to a higher charge density obtained with TAC coating due to its small molecular size which is confirmed by the observed higher EOF. The PVA coating in contrast give a hydrophilic and neutral surface with elimination of EOF. This coating is commercially available and commonly used for CE separations of peptides and proteins [73]. Contrary to the positively charged surfaces, the maximum peak efficiencies obtained with the PVA coating were achieved at the uppermost salt concentration tested and no limiting value was observed as for the other coatings. Moreover, the highest peak efficiency obtained was not dependent on the pH in the sample plug and peak stacking was still observed for all tested coatings. Thus, based on the obtained results, the observed peak stacking effect caused by adding salt to the protein sample is mainly due to a combined effect of both salt-surface and protein-salt interactions.
One concern with the obtained CE peak stacking effect was the difficulty of separating a mixture of large proteins with good resolution (data not shown). At a certain salt concentration, the charge-to-size ratio (and migration times) would be very similar for all proteins, regardless of their Mw. This will ultimately make all proteins co-migrate into one stacked peak, with reduced resolution. In order to solve such problems (improve resolution) and to get the analytical benefit and use of salt stacking effect for CE analysis, different strategies were investigated for large intact proteins. These involved using a second dimension separation technique to separate proteins based on their Mw after the salt stacking effect in the first dimension. This was evaluated by connecting a section (2-5 cm) of silica based monolith on-line to the CE coated capillaries close to the detector window. A silica based monolith with large through pore size was prepared in 50, 75 and 100 µm i.d capillaries [181] (Figure 18).

![Figure 18. SEM picture of silica based monolith in a 75 µm i.d. fused silica capillaries.](image)

The silica monolith was coated with M7C4I at high pressure. Typically, there are different factors controlling the resulted monolith and its morphology, such as, the type of monomer used, its concentration, its duration and the temperature of polymerization. However, some practical difficulties were encountered with the obtained monolith including high back pressure, difficulty in getting stable CE current and a continuous flow through the capillary, together with limited time. Therefore, no further investigations of the monolith could be conducted, but it would be interesting to perform such in future studies.
7 Analyzing Drugs in Biological Matrices

Qualitative or quantitative determination of drugs in various biological fluids is essential to understand the drug action and its distribution in the body. Drug analysis is also needed in biopharmaceutical development and quality control and assurance of pharmaceutical products. It is of great importance both clinically and pharmacologically as well as for many pharmaceutical industries to have efficient methods of measuring the active components in various matrices. Clinical monitoring of drug blood levels is required to provide the highest efficiency and safety for patients, especially for drugs with a relatively narrow therapeutic/toxic index, such as TCA drugs [182,183]. CE in combination with MS has frequently been used for pharmaceutical assays and for therapeutic drug assessments due to its speed, efficiency and versatility [110,184-186]. In Papers IV and Paper V, CE-ESI-MS was used to analyze and quantify TCA drugs and anticancer drugs (Imatinib) in human plasma respectively. The CE method was also evaluated for analysis of beta-blocker drugs (data not published).

7.1 Quantification of Tricyclic Antidepressant Drugs

Analysis of TCA drugs by liquid based separation is generally not straightforward, due to their hydrophobic nature and charge interactions. Such properties causes peak tailing, which in turn leads to poor reproducibility and limits the detection and quantification [187]. LC has widely been used to analyze TCA drugs in biological samples and pharmaceutical preparations [188]. However, the observed peak tailing and the use of different additives will, in most cases, complicate the on-line coupling with MS detection. In Paper IV, the optimal CE conditions for the analysis of TCA drugs and their major metabolites in human plasma were investigated. The major concern with the optimized CE conditions was their compatibility with on-line coupling to ESI-MS. The obtained results showed a significant improvement in peak shape and large reduction in the migration time of the analytes by adding acid and a high content of ACN to the TCA analytes (Figure 19).
The addition of both ACN and acid to the TCA samples, together with the use of M7C4I coated capillaries, yielded the highest peak efficiencies and the lowest variation in the migration time of the analytes. A consistent and dramatic improvement in peak shape and peak efficiency were observed by increasing the content of ACN added to the sample and this improvement was similar for all the TCA analytes tested (Figure 20).

Figure 20. The obtained peak efficiencies for the TCA analytes as a function of different percentages of ACN added to the samples, as presented in Paper IV.
Comparable improvements were achieved with the use of narrow i.d. (25 µm) capillaries. This is an interesting observation since adsorption of analytes to the capillary surface is generally increased with decreased the i.d. of the fused silica capillary. Thus, adding ACN to the TCA samples proved to effectively reduce their adsorption to the capillary wall, compared to adding Milli-Q water. This will subsequently improve the peak shape and peak efficiency and accordingly improve resolution and sensitivity. As a result, the method could readily be used for MS analysis since ACN as an organic modifier is often required in the sample to enhance the spray stability, as discussed in chapter 4. In addition, high repeatability in the migration time of the analytes was obtained together with significant improvement in peak efficiency and better detection and quantification limits. Thus, the obtained condition was used for quantification of the total fractions of TCA drugs in human plasma with CE-ESI-TOF-MS after SCX-SPE extraction (Figure 21).

![Figure 21. Extracted ion chromatograms of CE-ESI-TOF-MS analysis of the total fractions of imipramine (m/z 281.2) in human plasma, as presented in Paper IV.](image)

High specificity, sensitivity, linearity and repeatability were obtained for all the TCA samples with CE-ESI-TOF-MS analysis. The obtained results indicated that the method could readily be used for the analysis of TCA drugs and other related basic pharmaceutical drug molecules, either in biological samples or in pharmaceutical formulations. The optimal CE separation conditions for TCA drugs were thus further evaluated for their general applicability to other classes of basic drug molecules (Table 1).
Table 1. The selected TCA\(^1\), beta blocker\(^2\) and an anticancer\(^3\) drugs investigated.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Structure</th>
<th>Mw (g/mole)</th>
<th>pKa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imipramine(^1)</td>
<td><img src="image1.png" alt="structure" /></td>
<td>280</td>
<td>9.5</td>
</tr>
<tr>
<td>Desipramine(^1)</td>
<td><img src="image2.png" alt="structure" /></td>
<td>266</td>
<td>10.2</td>
</tr>
<tr>
<td>Clomipramine(^1)</td>
<td><img src="image3.png" alt="structure" /></td>
<td>314</td>
<td>9.5</td>
</tr>
<tr>
<td>Norclomipramine(^1)</td>
<td><img src="image4.png" alt="structure" /></td>
<td>301</td>
<td>9.4</td>
</tr>
<tr>
<td>Atenolol(^2)</td>
<td><img src="image5.png" alt="structure" /></td>
<td>266</td>
<td>9.4</td>
</tr>
<tr>
<td>Alprenelol(^2)</td>
<td><img src="image6.png" alt="structure" /></td>
<td>249</td>
<td>9.5</td>
</tr>
<tr>
<td>Esmolol(^2)</td>
<td><img src="image7.png" alt="structure" /></td>
<td>295</td>
<td>9.2</td>
</tr>
<tr>
<td>Carvedilol(^2)</td>
<td><img src="image8.png" alt="structure" /></td>
<td>406</td>
<td>7.7-7.9</td>
</tr>
<tr>
<td>Imatinib(^3)</td>
<td><img src="image9.png" alt="structure" /></td>
<td>493</td>
<td>1.5-8.0</td>
</tr>
</tbody>
</table>
7.2 Analysis of Beta-Blocker Drugs

The possibility of using the CE separation condition obtained for TCA drugs for its usefulness to other groups of drug compounds was further evaluated. Beta-blocker drugs (atenolol, alprenolol, esmolol and carvedilol) were investigated, as examples. Beta-blocker compounds are among the most commonly and widely used drug agents for various indications, mainly for the treatment of hypertension and other cardiovascular and respiratory disorders. Thus, it is of great interest to analyze/monitor such analytes either in biofluids or in pharmaceutical dosage forms using highly sensitive analytical tools. Although these analytes could easily be analyzed with LC, the CE condition was explored as an alternative or complementary technique. A comparable improvement in peak shape and peak efficiency was observed for all the studied beta-blocker drugs, as was achieved for the TCA drugs. This significant improvement is still largely related to the addition of a high content of ACN to the sample (data not published) compared to dissolving analytes in Milli-Q water (Figure 22).

![Figure 22. A) Peak efficiencies of beta-blocker analytes. B) Improvement in peak shape of atenolol as a function of different percentages of ACN added to the sample.](image)

Additionally, there is a considerable decrease in the migration time of the analytes observed, which could indicates and proves the potential of ACN to effectively reduce the analyte adsorption. This is an interesting finding since it shows the possibility of using CE for the analysis of different groups of basic pharmaceutical drug molecules and monitoring their blood level.
7.3 Quantification of Anticancer Drugs (Imatinib)

Imatinib (Gleeve® or Glivec®) is a recently introduced anticancer drug used mainly for the treatment of chronic myeloid leukaemia and gastrointestinal stromal tumor. It gives long-lasting response and prolonged survival rate and hence it is considered as the drug of choice for such diseases [189]. Therapeutic monitoring of the Imatinib blood level is therefore highly required for effective patient treatment and to ensure high therapeutic efficacy. In Paper V, the CE-ESI-MS analysis condition of Imatinib using two different extraction methods; LLE and SCX-SPE columns were investigated and compared using M7C4I coating. The optimal condition was then used for efficient extraction and quantification of Imatinib levels in patient plasma samples. High and repeatable extraction recoveries up to 91 % were obtained with SCX-SPE columns in comparison to recovery values between 30-35 % that was achieved using a LLE method. In addition, the CE-ESI-TOF-MS analysis was conducted in less than 10 minutes using acidic buffer with high peak efficiency, good peak shape and high repeatability in the migration time of the analyte (Figure 23).

![Figure 23. Extracted ion chromatograms of the CE-ESI-TOF-MS analysis of imatinib (m/z 494.2) on M7C4I coated capillaries as presented in Paper V.](image)

The method presented in Paper V showed high recovery, repeatability, linearity and sensitivity. Consequently, the method could readily be used for clinical assessments of the Imatinib blood level. The obtained results indicated also the great potential of CE-MS coupling for pharmaceutical analysis of a wide variety of basic drug molecules and their metabolites.
Capillary electrophoresis coupled to mass spectrometry has matured to the point of becoming an analytical technique of choice where the demands are fast measurements together with high resolving power. Nevertheless, further improvements and developments of all aspects of the technique are still required to enhance its usefulness as a technology for the routine analysis of challenging analytes such as large proteins, the digest of complex samples and drugs in biological matrices. Additionally, many improvements are needed to overcome the various difficulties in achieving high stability, reproducibility and sensitivity over long-term operation. This thesis describes the development of such CE methods coupled on-line to ESI-MS.

Analyte-wall interactions have long been known to cause problems in CE analysis, especially with increase in analyte size, basicity and complexity. This can be avoided using surface modified capillaries, taking into consideration the importance of stable and high EOF for electrospray operation in ESI-MS. In Papers I and II, the M7C4I and PVPy-Me coatings respectively, were investigated for the first time as silica surface modifiers for rapid CE-ESI-MS separation of peptides, proteins and the digest of complex samples to prevent analyte-wall adsorption. High MS stability and successful CE-ESI-MS separation was obtained with both coatings. The M7C4I coating showed good stability over a pH range of 2-10 and also long life stability for 3 days of continuous use without recoating steps. Additionally, the M7C4I coating showed a particular advantage in that it could handle large intact proteins up to 669 kDa with extremely good peak shape and efficiency. Surprisingly, this work is the first report of CE analysis of extremely large intact proteins with high peak efficiencies.

An unexpected peak stacking effect was obtained by adding alkali salts to the protein samples and this was demonstrated in Paper III, where protein samples with a wide range of molecular sizes (14-669 kDa) were analyzed with various surface modified capillaries. The optimal concentration of alkali salts needed in the sample plug varies depending on the surface coating used, the protein Mw and its molar concentration. In Papers IV and Paper V, the M7C4I was further investigated for rapid CE-ESI-MS analysis and quantification of pharmaceutical drug molecules in biological samples. Besides, it was evaluated for monitoring of the drug level important during therapy processes to ensure the highest therapeutic efficacy and response. The obtained results showed great potential of the method for the efficient
analysis of such analytes together with low sample consumption and a fast analysis time comparable to other analytical techniques.

The combination of CE with ESI-MS faced remarkable development over the years and this hyphenated technique should have a bright future. The major direction for further development is to promote the availability of a robust and sensitive method for this hyphenation. Since there is a growing interest in analysing large intact proteins and post-translational modifications, taking advantage of CE to handle large proteins as demonstrated in this thesis should be considered. Thus, further studies regarding the underlying mechanism behind the observed positive salt effect on CE analysis of intact proteins should be conducted. Furthermore, a more thorough evaluation of the factors controlling the CE separation efficiency of intact proteins is needed. The use of multidimensional coupling of CE is important, especially for the analysis of intact protein with high resolution. Moreover, this is important for the analysis of complex samples as it has the possibility of in-capillary sample pre-treatment, which in turn allows for better sensitivity and reproducibility. The potential of using M7C4I as surface modifications for CE-MALDI-TOF/TOF-MS and CE-ESI-MS/MS analysis of proteins in clinical and biological samples needs to be explored further, taking advantage of the rapid coating process, ease of regeneration and long life stability. This coupling is promising also for routine pharmaceutical and biomedical applications, such as screening for protein biomarkers, characterizing protein drugs and clinical monitoring of drug levels and their metabolites.

“Praise be to Allah who guided us to this, nor do we have found guidance for the fact that Allah guided”

الحمد لله الذي هدانا لهذا وماكنا لهداة لولا أن هدانا الله
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Anisa Elhamili

January 27th, 2011, Uppsala, Sweden
Kapillärelektrofores (CE) har på senare tid uppnått en högre mognadsgrad som analytisk teknik i en värld där det ställs höga krav på korta analystider och hög upplösning. Vidare förbättringar och utveckling av tekniken är dock fortfarande nödvändiga för att kunna tillämpa den inom rutinanalys av komplexa molekyler och prover så som stora proteiner, digerat av humana kroppsvätskor eller läkemedel i biologiska matriser. Förbättringar av CE tekniken behöver också göras för att kunna uppnå bättre stabilitet, reproducerbarhet och känslighet under långtidsanvändning. I denna avhandling beskrivs utveckling av sådana CE metoder kopplade till masspektrometri (MS).

goda möjligheter till sådana analyser med minimal provåtgång och snabb analyser jämfört med andra tekniker.

Kopplingen av CE till ESI-MS i ett system, har under senare år utvecklats avsevärt och förväntas också gå en ljus framtid till mötes. Den framtida utvecklingens huvudsakliga in riktning är mot mer robusta och känsliga metoder. Det finns också ett ökande intresse för analys av stora intakta proteiner bland annat för att kunna studera post-translationella modifieringar och CE möjliggör då analys av stora proteiner som också visats i denna avhandling. Det är av stor vikt att vidare studera de underliggande mekanismerna av höga saltalters effekt och påtagliga fördelar inom analys av stora intakta proteiner. Dessutom behövs en djupare utvärdering av de faktorer som styr separationseffektiviteten i sådana sammanhang. Även användning av CE i multidimensionella separationer kan vara avgörande när en högre upplösning av intakta proteiner vill uppnås. Framförallt är detta viktigt vid analys av komplexa prover därför att det då ges möjlighet att också upparbeta provet i kapillären vilket i sin tur resulterar i bättre känslighet och reproducerbarhet. Möjligheterna vid användning av M7C4I ytan för CE-MALDI-TOF/TOF-MS och CE-ESI-TOF-MS analys av proteiner i kliniska och biologiska prover behöver också vidare undersökas där fördelarna av snabb ytmodifieringsproceduren, lätt ytregistrering och yttans långtidsstabilitet kan utnyttjas. Ytterligare möjligheter vid användning av ett sådant system kan dessutom finnas inom rutinanalys för farmaceutiska och biomedicinska tillämpningsområden så som karakterisering av proteinläkemedel och i jaleten på nya proteinbiomarkörer.
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