Capillary electrophoresis – mass spectrometry using noncovalently coated capillaries for the analysis of biopharmaceuticals

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Capillary electrophoresis – mass spectrometry using noncovalently coated capillaries for the analysis of biopharmaceuticals

Capillaire elektroforese – massaspectrometrie
gebruikmakend van niet-covalent gecoate capillairen
voor de analyse van biofarmaceutica

(met een samenvatting in het Nederlands)

Proefschrift

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Contents

Chapter 1

Introduction

1. General remarks

Biologics are medicinal products that are derived from living organisms. In contrast to most conventional drugs, they are produced by biological processes and not by chemical synthesis. Since the late 19th century, biologics have been introduced as a revolutionary new way to cure and prevent diseases. Among the first biologically derived therapeutics were vaccines against cholera, typhoid, and the plague, which had an enormous impact on public health and life expectation. In the following decades, peptides and proteins isolated and purified from animal tissues, like insulin, were introduced as drugs. The development of protein pharmaceuticals strongly grew and matured by the introduction of recombinant DNA technology in the early 1970's. The first so-called biopharmaceutical made available via biotechnology was human insulin, which was allowed to the public market by the Food and Drug Administration (FDA) in 1982. Since then, the production of proteins for therapeutic purposes has opened new pathways for the treatment of a variety of chronic and life-threatening diseases. Nowadays, the assortment of biopharmaceuticals includes vaccines, blood components, therapeutic proteins and gene therapeutics. Until 2009, over 300 biopharmaceutical products have been approved by the FDA [1] and as of July 2008 more than 600 are in clinical trials for treatment of *e.g.* cancer, infectious diseases, autoimmune diseases, and HIV [2].

As for any drug for human use, biopharmaceuticals have to meet stringent quality requirements in order to assure efficacy and safety. However, due to their macromolecular nature, the full characterization of biopharmaceuticals in terms of parameters such as identity, content, purity, stability, conformation, and function poses a great challenge [3]. Moreover, multi-step biotechnological production processes may show variability, introducing product diversity and isoforms which can affect activity. Usually a large number of techniques has to be applied to gain information on the various properties of a pharmaceutical protein. When it comes to assessment of *e.g.* impurities, degradation products, or glycoforms, biopharmaceutical characterization presents itself as a mixture analysis problem. That is, specific and reliable detection of individual sample components usually requires a preceding separation step. Clearly, biopharmaceutical characterization strategies would benefit from an approach in which efficient separation and selective spectrometric detection are combined in an on-line fashion.

Over the last decades, mass spectrometry (MS) has developed into one of the most popular and useful detection techniques for the characterization of recombinant proteins in the biotechnology development area [4]. Matrix-assisted laser desorption ionization (MALDI) in essence is well suited as an ionization technique for MS of intact proteins. However, as protein ionization has to take place from a surface after addition of a light-energy absorbing matrix, its coupling with liquid-phase separation techniques is less straightforward. For electrospray ionization (ESI) compounds need to be in a solution which is sprayed into a fine mist of charged droplets from which ions are produced. This makes it in principle well

compatible with separation techniques providing a liquid effluent. ESI of full proteins provides multiple charged ions giving rise to charge-state distributions with *m/z* values typically between 500 and 3000. These are within the measurable range of most commercially available mass analyzers. Protein molecular masses can be obtained by deconvolution of the recorded ESI mass spectra. ESI is a soft ionization method which does not induce protein fragmentation – and even may leave the conformation of the analyzed protein unchanged – [5], which can be important when structural integrity of intact proteins, like *e.g.* biopharmaceuticals, has to be probed.

The combination of liquid chromatography (LC) and MS has proven a very powerful tool for the analysis of low-molecular-weight drugs and biomolecular compounds, such as metabolites and peptides. LC is an established technique for the separation of proteins based on their molecular mass (size exclusion), charge (ion exchange) or hydrophobicity (reversed phase and hydrophobic interaction) [6]. LC is easily automated and can be applied routinely. However, it may not be easy to achieve high-resolution separations of protein species by LC, especially when the analytes are highly similar. Moreover, adverse and/or irreversible interactions of proteins with the LC stationary phase and denaturation of proteins in mobile phases containing high organic modifier concentrations can seriously diminish LC performance and decrease separation efficiencies.

Being multiply-charged compounds, proteins can be efficiently separated by electrophoretic techniques. Polyacrylamide gel electrophoresis (PAGE) is a widely used technique for the analysis of proteins [7]. In the one-dimensional format it normally provides efficient size-based separations. However, PAGE is frequently not suited for the discrimination of highly related protein species such as degradation products and glycoforms. Resolution can be improved using two-dimensional gel electrophoresis (2D-GE) involving an isoelectric focusing (IEF) step. Although multiple samples can be run on the same gel, 2D-GE is labor-intensive and has very long analysis times. Furthermore, it cannot be coupled on-line to MS and lacks precise quantitation.

Capillary electrophoresis is an attractive tool for the separation of biotechnology-derived proteins offering efficient and fast separations [8]. Capillary isoelectric focusing (CIEF) is currently used to probe charge heterogeneity of pharmaceutical proteins. Capillary gel electrophoresis (CGE) provides size-based separations and is used in biopharmaceutical analysis to monitor the presence of subunits or fragments of proteins such as monoclonal antibodies. However, due to the use of relatively high concentrations of nonvolatile buffer components such as ampholytes (CIEF) and gel polymers (CGE), combination of these techniques with MS is quite problematic. In this respect, capillary zone electrophoresis (CZE) offers much better possibilities. CZE can provide highly efficient separation of proteins based on their charge-to-size ratio. This means that changes in protein charge – resulting *e.g.* from chemical degradation or post-translational modification – or changes in protein size and shape – as result of aggregation or denaturation – in principle can be probed by CZE. Furthermore,

CZE can be conducted in simple aqueous buffers under conditions that allow detection of proteins in their native state. Separation conditions can be optimized effectively by changing the nature, concentration, and pH of the background electrolyte (BGE), and volatile BGEs can be selected to facilitate coupling with MS.

As high-molecular-weight species like proteins have relatively low diffusion coefficients, in principle very high plate numbers can be obtained for these compounds in CZE. However, an important challenge encountered in CZE of proteins is their tendency to adsorb to the fused-silica capillary wall [9]. Adsorption may seriously deteriorate separation efficiencies and – when irreversible – can lead to protein losses. Protein adsorption in principle can be avoided using BGEs of extreme pH and high ionic strength. However, the applicability of these approaches may be limited due to protein stability problems and their incompatibility with MS detection. The more common approach in CZE to prevent protein adsorption is to coat the capillary wall with non-adsorptive agents. An elegant and simple way to create effective coatings is by applying charged polymers to the fused-silica surface [10]. These coatings can be prepared straightforwardly by rinsing the capillary with an aqueous solution of a positively charged polymer that electrostatically adsorbs to the negatively-charged capillary wall. When required, a second layer of a negatively charged polymer can be applied. It has been demonstrated that multiple layers (three of more) of oppositely charged polymers even result in more stable CZE systems. By properly choosing the charge of the last polymer layer in combination with a BGE of the right pH, efficient CZE systems suitable for the analysis of acidic or basic proteins can be created.

Coupling of CZE with MS provides a potentially powerful tool for protein analysis. CZE-MS cannot only be highly useful for purity and stability analysis, but also for the characterization of closely resembling protein species. Currently, the predominant way to couple CZE with MS is through ESI using a sheath-liquid interface [11]. The CZE and ESI processes require closed electrical circuits having a common electrode at the capillary outlet. The sheath liquid facilitates the electrical contact required for both CZE and ESI. CZE-ESI-MS analysis of intact proteins and their modifications required employment of mass analyzers of high resolution and high mass accuracy, like time-of-flight (TOF) analyzers. The strong technological developments in TOF instrumentation and the much wider commercial availability of affordable TOF-MS instruments over het last decade [12] provides good opportunity for CE-MS of intact proteins.

2. Scope and outline

At the start of this project, the utility of CE-TOF-MS for the analysis of biopharmaceuticals had been hardly explored. In order to establish the full potential of CE-TOF-MS in this field, efficient and reproducible protein separations are required. Therefore, the utility of capillaries noncovalently coated with charged polymers was studied. From earlier work, the performance of polymeric bilayer coatings of Polybrene-(polyvinyl sulfonic

acid) for the analysis of acidic proteins was known. However, the utility of multilayered coatings for CE-MS analysis of basic proteins had not yet been evaluated systematically. In this thesis, emphasis was on development of CE methods for basic proteins, with particular attention for the positively charged Polybrene-dextran sulfate-Polybrene (PB-DS-PB) coating. These coatings were prepared by successive flushes with solutions of the charged polymers, leading to formation of electrostatically adsorbed layers. The performance and stability of the triple-layer coating was evaluated for basic proteins analyzed at medium and low pH. The suitability of the triple-layer coating for CE-TOF-MS was studied, paying attention to compatibility. CE-MS coupling was achieved via a sheath-liquid interface, and optimum interfacing conditions were investigated. A disadvantage of this type of interfacing is that the sheath liquid dilutes the CE effluent leading to reduced detection sensitivity. In order to avoid this drawback, the performance of a prototype sheathless interface for intact protein analysis by CE-MS was investigated and compared to sheath-liquid CE-MS. The potential of the developed CE-TOF-MS systems was evaluated by analyzing various pharmaceutical proteins, addressing issues such as purity, stability, heterogeneity, and product composition.

An overview of the use of CE-MS in the field of intact protein analysis is presented in **Chapter 2**. Section 2.1 treats the CE separation modes, capillary coatings, and preconcentration techniques that have been applied so far in the field of intact protein analysis. Furthermore, various CE-MS interfaces and MS analyzers employed for protein analysis are described. The applicability of CE-MS in protein analysis is illustrated by examples including biomarker discovery, glycoprotein profiling and proteomics approaches. In Section 2.2 technological developments and new applications of CE-MS for intact proteins in the period 2007-2010 are discussed.

In **Chapter 3** the effectiveness of charged noncovalent polymer coatings in minimizing adsorption of basic proteins is studied by evanescent-wave cavity ring-down spectroscopy (EW-CRDS). A single layer of PB and a triple PB-DS-PB coating applied to the surface of a silica EW-CRDS prism are evaluated. Comparative experiments with CE-UV are performed using a fused-silica capillary without and with the respective coatings. Data on the magnitude of the EOF, peak area, electrophoretic mobility, and peak width are interpreted with respect to protein adsorption and discussed in conjunction with the EW-CRDS measurements.

Chapter 4 reports on the potential of capillaries with a noncovalent PB-DS-PB coating for the analysis of basic proteins by CE-UV. The repeatability, stability and CE performance of the coating is evaluated using model proteins. The applicability of the CE-UV system is studied by the profiling of immunoglobulin G_1 at medium pH, and the analysis of basic llama antibodies and their binding products at low pH. The feasibility of using the triple-layer coating combined with time-of-flight mass spectrometry (TOF-MS) for protein analysis is investigated by the characterization of a llama antibody sample.

CE-MS of intact basic proteins using PB-DS-PB coated capillaries is studied and further optimized in **Chapter 5**. The compatibility of the triple layer coating with ESI-MS is investigated, and the sheath liquid and BGE composition are optimized with respect to protein MS response. The capability of the PB-DS-PB system to separate and characterize basic proteins is tested with a mixture of model proteins and acetylated lysozyme, both representing a mixture of highly similar protein species. The repeatability, response curves and limits of detection of the CE-MS method are evaluated

The applicability of CE-TOF-MS using noncovalent capillary coatings for the analysis of biopharmaceuticals is studied in **Chapter 6**. PB-PVS and PB-DS-PB capillary coatings are used for the CE-MS analysis of the drugs recombinant human growth hormone (rhGH), oxytocin, and recombinant human interferon-β-1a (rhIFN-β). The potential of the PB-PVS and PB-DS-PB CE-MS systems to separate and identify protein degradation products is investigated by the analysis of heat-exposed samples of rhGH and oxytocin. The capability of the PB-DS-PB CE-TOF-MS method to assess the glycoform heterogeneity of pharmaceutical proteins is tested by the CE-MS analysis of rhIFN-β.

Chapter 7 describes a study into the performance of a prototype porous tip sprayer for sheathless CE-MS of intact proteins. The performance of the sheathless interface is first tested using a conventional ESI source. With a mixture of model proteins, parameters such as migration time, reproducibility, response linearity and limits of detection are investigated. Results are compared with sheath-liquid CE-MS interfacing. Special attention is paid to the role of organic solvent in the sheath liquid. Finally, the sheathless CE-MS system is tested in conjunction with a nanoESI source in order to further improve detection limits.

In **Chapter 8** sheathless CE-TOF-MS employing the porous tip sprayer is used to characterize preparations of drug-lysozyme conjugates. Drug-lysozyme products had been prepared by first coupling BOCmethionine to lysozyme followed by conjugation with kinase inhibitors LY364947, erlotinib and Y27632 via a platinum-based linker. CE-TOF-MS is used to separate the individual conjugates and assign their identity based on the measured molecular weights. Drug-protein stochiometries are derived and drug loading values are determined.

Chapter 9 provides some general conclusions and comments on the CE-MS systems developed for the analysis of biopharmaceuticals. Also perspectives and recommendations are presented.

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Chapter 2

Capillary electrophoresis – mass spectrometry of intact proteins: an overview

2.1

Principles and applications

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Abstract

Developments in the fields of protein chemistry, proteomics and biotechnology have increased the demand for suitable analytical techniques for the analysis of intact proteins. In 1987, capillary electrophoresis (CE) was combined with mass spectrometry (MS) for the first time and shortly after its potential usefulness for the analysis of intact (*i.e.* non-digested) proteins was shown. This article provides an overview of the applications of CE-MS within the field of intact protein analysis. The principles of the applied CE modes and ionization techniques used for CE-MS of intact proteins are shortly described. It is shown that separations are predominantly carried out by capillary zone electrophoresis and capillary isoelectric focusing, whereas electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI) are the most popular ionization techniques used for interfacing. The combination of CE with inductively coupled plasma (ICP) MS for the analysis of metalloproteins is also discussed. The various CE-MS combinations are systematically outlined and tables provide extensive overviews of the applications of each technique for intact protein analysis. Selected examples are given to illustrate the usefulness of the CE-MS techniques. Examples include protein isoform assignment, single cell analysis, metalloprotein characterization, proteomics and biomarker screening. Finally, chip-based electrophoresis combined with MS is shortly treated and some of its applications are described. It is concluded that CE-MS represents a powerful tool for the analysis of intact proteins yielding unique separations and information.

1. Introduction

Developments in the fields of protein chemistry, proteomics and biotechnology have caused an increasing demand for sensitive and selective analytical tools for the analysis of intact proteins. Today, various separation techniques are available for the qualitative and quantitative analysis of proteins. Frequently used techniques are slab-gel electrophoresis (SGE), liquid chromatography (LC) and capillary electrophoresis (CE) [1]. SGE is an established technique for protein separation, as many samples can be analyzed simultaneously and a relatively large number of proteins can be resolved applying two-dimensional SGE. However, limitations are the relatively long and labor-intensive analysis, the necessity of off-line detection, and the lack of precise (and automated) quantitation. LC is advantageous due to its separation power, ease of automation and routine coupling with various detection principles, like mass spectrometry (MS). On the other hand, adverse interactions of proteins with the stationary phase and denaturation of the proteins in organic mobile phases can seriously hinder LC analyses and decrease separation efficiency and resolution. CE offers attractive features for the analysis of proteins, as the analysis times can be relatively short and only minute amounts of sample are needed. Furthermore, CE analyses are carried out in fused-silica capillaries under aqueous conditions and in the absence of a stationary phase. This enables the study of proteins without causing conformational changes due to organic modifiers and/or a stationary phase. In addition, separation conditions in CE can be chosen in such a way that separations can be performed under (near-)physiological conditions avoiding protein degradation during analysis. Finally, as the CE separation is a function of charge, size and shape of a compound, small differences in the size or charge of proteins may be sufficient for separation, especially with the high efficiencies normally obtained in CE.

MS has developed into one of the most popular and useful detection techniques in separation sciences. Coupling of CE with MS is less straightforward than LC-MS coupling, but nevertheless since the introduction of CE-MS in the late 80's of the past century [2,3], MS has gained importance as a selective detection technique for CE. MS considerably enhances the utility of CE by providing information about the identity of the separated compounds. It is well suited for protein analysis because of its sensitivity, selectivity and its ability to identify proteins and to determine their primary structures. In a proteomics setting, proteins are normally characterized by first digesting the protein(s). Thereafter, the formed peptides are separated by LC or CE and analyzed with MS/MS techniques. With the aid of the gained peptide masses and databases, the proteins in a mixture can then be identified. If protein identification is the main issue, this approach is most-suited and often gives excellent results [4]. However, in protein analysis elucidation of the primary structure may not always be the primary goal, and the analysis of the intact (*i.e.* non-digested) protein(s) can be necessary in order to gain additional information. Profiling of protein samples can, for example, be required during quality control of pharmaceutical proteins. CE-MS can be used to determine the purity of a product: sample constituents can be separated and the obtained mass spectra

give tentative information about the identity of the main compound and its impurities. The same holds for the stability analysis of proteins: by analyzing the protein in time, the degradation can be monitored by CE-MS and structure modifications can be determined. Impurities and degradation products may be strongly related to the protein but can, for example, also origin from the production process. Another interesting application of CE-MS is the characterization of naturally occurring isoforms. Mixtures of isoforms, like glycoforms, can be complex, but with the aid of CE-MS, these can be (partially) separated and from the MS information, differences in structure can be revealed. In addition, analyzing intact proteins by CE-MS may even yield information about the way proteins are folded, as changes in folding can induce changes in electrophoretic mobility and character of the mass spectrum. Another interesting application of CE-MS is the analysis of noncovalent protein complexes and the monitoring of the involved interactions.

The abovementioned subjects indicate that there is a need for techniques that allow intact protein analysis in order to obtain biological, biochemical and pharmaceutical information. As both CE and MS are increasingly important tools for protein analysis, it is not surprising that a considerable number of papers reporting on the development and application of CE-MS for intact protein analysis have appeared over the last years. In the present article, an overview of these systems is given. The paper is confined to CE-MS studies in which entire ("intact") protein molecules are analyzed, that is, in which protein chains are not deliberately broken down prior to analysis. However, protein modifications as results of *e.g.* (natural) degradation or glycosylation are covered by the review. In the first section the most frequently CE modes and some general aspects concerning CE-MS analysis of intact proteins will be described. Additionally, capillary coatings and possibilities for sample preconcentration in CE will be shortly discussed. Furthermore, the most commonly applied ionization techniques and mass analyzers will be treated from the viewpoint of intact protein analysis. Subsequently, intact protein analysis using capillary zone electrophoresis and capillary isoelectric focusing in combination with electrospray ionization MS, matrix-assisted laser desorption ionization MS and inductively coupled plasma ionization MS will be systematically discussed, supported by tables and illustrative examples. A final section focuses on a relative new field: chip-based electrophoresis combined with mass spectrometry for intact protein analysis.

2. CE of proteins

2.1 CE modes

The separation of proteins can be performed using various CE modes, such as capillary zone electrophoresis (CZE), capillary isoelectric focusing (CIEF), micellar electrokinetic capillary chromatography (MEKC) and capillary gel electrophoresis (CGE). For combining CE with MS, CGE and MEKC are less suitable, due to the presence of

nonvolatile separation media (*i.e.* polymers and surfactants) [5]. Until now CE-MS analyses of intact proteins predominantly have been carried out using CZE and CIEF.

The most frequently used mode in CE-MS of proteins is CZE. In this CE mode, the capillary is merely filled with a background electrolyte (BGE) and the separation is accomplished by differences in the electrophoretic mobility of the analytes. With CZE in principle high separation efficiencies can be obtained, as longitudinal diffusion is ideally the only source of band broadening. As diffusion coefficients of proteins are relatively small, efficiencies in terms of theoretical plates can reach up to one million or more. Simple buffers can be used as BGE and electrophoretic mobilities can be manipulated by changing the pH and/or the ionic strength of the BGE and by addition of ion pairing or complexing agents to the BGE. Buffers like phosphate and borate buffers are frequently used in CZE, but organic buffers, such as TRIS, can also be applied as BGE. With these BGEs, (near-)physiological conditions can be mimicked and proteins can be studied in their natural state. Next to these nonvolatile buffers, also volatile electrolytes can be used, which is important from the viewpoint of the compatibility of CE with MS.

The second popular CE mode for separating proteins is CIEF. In CIEF, amphoteric compounds, like proteins, are separated according to their isoelectric point (pI). In order to achieve that, the sample is mixed with a mixture of ampholytes of slightly different p*I* spanning the desired pH range, and this mixture is loaded into the capillary. Under influence of an electric field, a pH gradient is formed by the ampholytes along the capillary, while the proteins migrate according to their electrophoretic mobility. When the proteins reach the point in the capillary in which the pH equals their pI, they become net uncharged and stop migrating. After this focusing step, the analytes are forced into the mass spectrometer, by applying a pressure to the capillary inlet (*i.e.* the mobilization step). An advantage of CIEF is that the zones containing a certain protein will be constantly focused, as a protein molecule that enters a zone of different pH will become charged and migrates back. Furthermore, as the entire capillary can be filled with sample, concentration limits of detection are often lower than with other electrophoretic methods. On the other hand, the mobilization step introduces band broadening, which can be minimized by applying an electrical field during mobilization.

2.2 Capillary coatings

A typical problem in CE of proteins is that the separation is often hampered by adsorption of the proteins onto the fused-silica capillary wall due to electrostatic and hydrophobic interactions. To prevent this adsorption, BGEs with an extremely low or high pH can be used. However, most proteins may be unstable at these pH values. Furthermore, in CZE it is desirable to be able to tune the pH of the BGE so that separations can be optimized. In CIEF, obviously, the pH must be varied along the capillary to enable the separation. Furthermore, in CIEF it is desirable to suppress or minimize the electroosmotic flow (EOF) to allow complete focusing before the proteins are mobilized. Therefore, in order to allow

efficient CZE and CIEF separations of proteins, approaches have been designed to minimize or completely prevent protein-wall interactions and to modify the EOF.

Over the past two decades, different types of capillary coatings have been developed in order to facilitate protein CE. Excellent reviews describing and discussing these coatings are available [6-13]. For CE-MS of proteins, coatings should be either covalently attached or physically adsorbed onto to the capillary wall, so that the coating agents do not migrate into the mass spectrometer. Covalent coatings often make use of a reactive silane to attach the coating agent onto the capillary wall. The advantage of covalent attachment is the high stability and lifetime of the coating, but the reaction schemes involved often are complex and labor intensive. Therefore, they are not always easily applicable and reproducible, and differences between capillaries are not an exception. Noncovalent coatings make use of physical adsorption caused by strong electrostatic interactions between the coating agent and the capillary wall as the main force of attachment. Noncovalent coatings may be added to the BGE, but as this could cause ionization suppression, in CE-MS they are applied by simply flushing the capillary with the solution of the coating agent prior to CE-MS analysis. Afterwards, residual coating solution is easily removed via a rinsing step. Various coatings have been applied in protein analysis by CE-MS, such as cellulose derivatives [14,15], surfactants [16], polystyrene nanoparticles [17], polyamines [18,19] and polymers like polyacrylamide [20], EPyM-DMA [21], Polybrene [22], Polybrene-poly(vinyl sulfonic acid) bilayers [23] and AAMPS [24]. These coatings often result in a suppressed [14,15,20] or reversed EOF [16-19,21,22]. All coatings show good stability over prolonged periods of time, but in some cases additional flushing with coating agent in between runs is required to maintain the coating performance [18,19,21,23]. Most of these coatings are stable over a wide pH range [18,21,23], however, the workable pH-range is limited as the coating and analyzed proteins preferably should have the same charge in order to avoid protein-wall interactions. The effect of a capillary coating on protein separation is clearly illustrated in Figure 2.1. With a bare fused-silica capillary using an ammonium acetate buffer (pH 5.5), only cytochrome *c* can be discerned, whereas lysozyme and ribonuclease A are not detected. However, coating the capillary with EPyM-DMA enables baseline separation of the three proteins under the same BGE conditions and allowing their MS detection and identification [21].

2.3 Preconcentration techniques

In CIEF the entire capillary is filled with sample and low limits of detection may be obtained. In CZE analysis the limits of detection can be relatively poor due to the limited amount of sample which can be introduced into the capillary. Therefore, several strategies have been developed to preconcentrate samples before CZE analysis. Large volume sample stacking (LVSS) has been applied in CZE analysis of proteins to improve the loadability [25,26]. In LVSS of acidic proteins a relatively large plug of low conductivity sample with a pH above the p*I* of the proteins is introduced into the fused-silica capillary, which has been filled with a

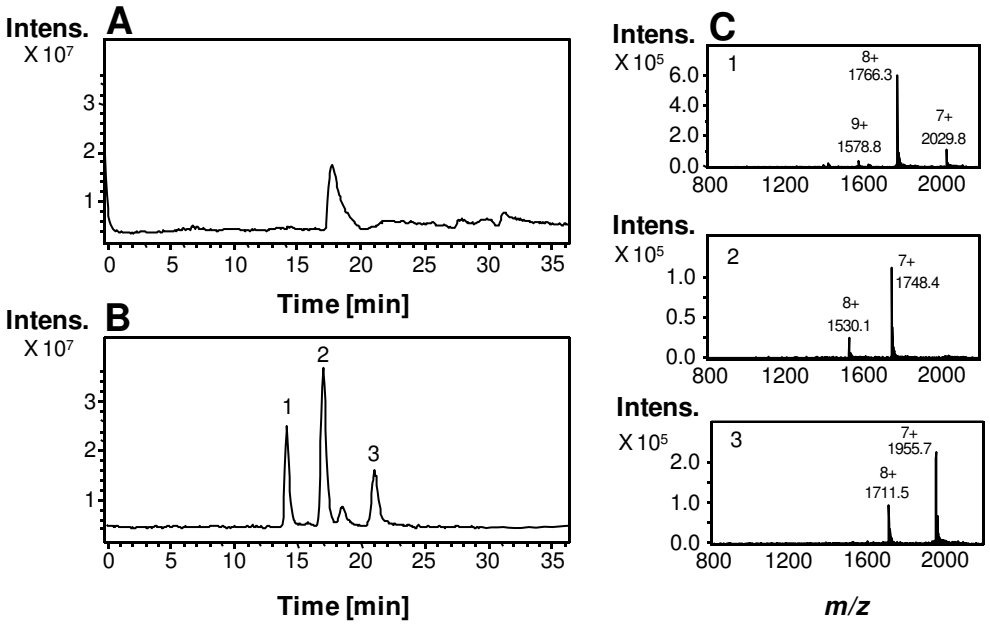

Figure 2.1. Total ion electropherograms obtained using CZE-ESI-MS of a mixture of the basic proteins lysozyme (1), cytochrome *c* (2), and ribonuclease A (3) using a BGE of ammonium acetate buffer at pH 5.5 and (A) a bare fused-silica capillary or (B) an EPyM-DMA-coated capillary. (C) Mass spectra of the main peaks obtained with the EPyM-DMA-coated capillary [21].

BGE of relatively high conductivity before. Then, a negative voltage is applied and the EOF leads the large plug to move in the direction of the capillary inlet, hence pushing this plug out of the capillary. Meanwhile, the negatively charged proteins are subjected to a strong local electric field and consequently move with a high electrophoretic velocity towards the boundary between the sample plug and the BGE zone. Once the proteins reach this boundary, their velocity is slowed down in the lower electric field of the higher conductivity buffer, resulting in the stacking of the analytes. When the main part of the sample plug is removed and most of the proteins are concentrated in a narrow zone, the voltage polarity is switched to positive and the separation starts under normal CE conditions. In principle the same procedure can be used for basic proteins (positively charged), but now a positive stacking voltage should be applied and the capillary wall should be positively charged (by using an appropriate coating) in order to reverse the EOF. Typical concentration factors which routinely can be obtained with LVSS in CE-MS are around 10 (Figure 2.2). An isotachophoresis (ITP) preconcentration step before the CZE separation can also be performed to increase loadability of proteins [27-30]. The ITP step takes place in a discontinuous electrolyte system consisting of a leading electrolyte of high mobility and a termination electrolyte exhibiting low mobility. Proteins, which are introduced in a relatively large injection volume, with mobilities between the two electrolytes will be focused during the CZE separation step to form narrow, neighboring zones. The most commonly applied leading and terminating electrolytes in protein CE-MS analysis are ammonia and acetic acid, respectively. In CE-MS of proteins with ITP preconcentration, injection plugs of 15 to 45% of the capillary volume have been reported

[28,30], leading to concentration factors between 10 and 50. Solid-phase extraction (SPE) is another way to achieve preconcentration of proteins in CZE [22,31-34]. The micro-SPE cartridge, filled with *e.g.* C_{18} or C_8 reversed phase material, is inserted at the inlet side of the CE capillary, allowing the loading of a relatively large sample volume. Proteins are trapped on the SPE material and matrix components do not or are removed via a rinsing step. After preconcentration, the proteins are desorbed in a small volume into the CE capillary and will be separated by CZE. With SPE-CE-MS, concentration factors of at least 100 can be obtained. It should be noted that so far all SPE-devices used in protein CE were fabricated in-house and that carrying out on-line SPE-CE-MS may not be a trivial procedure.

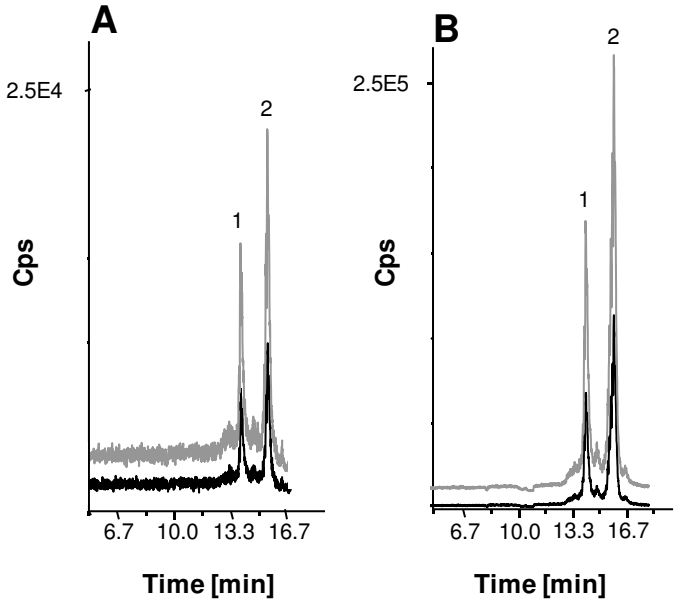

Figure 2.2. (A) CZE-ICP-MS analysis of a mixture of the rabbit liver metallothioneins MT1 (1) and MT2 (2) without an LVSS preconcentration step and (B) with an LVSS preconcentration step. The proteins are detected by their cadmium content as monitored with ICP-MS. The upper trace represents the ^{114}Cd isotope and the lower trace the ^{111}Cd isotope [26].

3. CE-MS coupling for proteins

3.1 Ionization techniques and interfacing

In CE-MS, the outlet of the separation capillary has to be connected to the ion source of the mass spectrometer, and obviously, no outlet buffer vial can be used. Still, CE requires a closed electrical circuit and, therefore, a CE-MS interface should provide a means to apply voltage to the capillary outlet. The combination of CE with MS detection started around two decades ago, when Smith *et al.* [2,3] introduced the first working interface. Since then, various interfaces for several types of ionization sources have been developed. For CE-MS of proteins, so far electrospray ionization (ESI), matrix assisted laser-desorption ionization (MALDI) and inductively coupled plasma ionization (ICP) have been used.

3.1.1 Electrospray ionization

ESI is the main ionization technique used for CE-MS of proteins. In ESI, a fine spray of charged droplets is formed at the capillary tip from which the solvent solution flows by means of a high voltage between the tip and the inlet of the mass spectrometer. The solvent evaporates, causing shrinkage of the charged droplets and disintegration of the drops finally leads to the formation of gas-phase ions. Due to the high charge density in the droplets, proteins tend to give multiply charged molecular species, giving rise to the so-called charge envelop in the protein mass spectrum consisting of a number of peaks with mass-over-charge (*m/z*) values typically between 500 and 3000. Multiple charging allows for proteins to be detected in mass ranges much lower than their molecular mass and, therefore, ESI-MS of proteins in principle is also possible with quadrupole and ion trap mass analyzers. Protein molecular masses are obtained by deconvolution of ESI mass spectra, and shifts in protein molecular mass may indicate a modification, like lactosylation [35] or glycosylation [36]. The shape and position of the charge envelop in principle can also provide information on the conformation of proteins [37,38].

Interfacing CE with MS via an ESI source can roughly be performed in two different ways, as described more extensively in several recent reviews [39-42]. In the first approach, an additional liquid is used to apply the terminating CE voltage to the BGE. This so-called sheath-liquid interface (Figure 2.3A) is the most widely used interface for CE-MS. In the sheath-liquid interface the CE capillary is surrounded by a second capillary in a coaxial arrangement. Through this second capillary a sheath liquid (typical flowrates, 2 to 5 μL/min) is led, which merges with the CE effluent at the capillary outlet. The terminating CE voltage is applied to the sheath liquid to close the electrical circuit. Often, a third coaxial tube delivers a gas flow, which aids nebulization into the ESI source. The most important feature of this interface type for protein analysis is the sheath-liquid composition and its flow rate [43-45]. It has been shown that the signal intensity and the charge envelop may shift under influence of the pH, electrolyte concentration and type and concentration of organic modifier. In the second CE-ESI-MS approach, the voltage is directly applied to the CE buffer. This can be achieved by applying a metal coating to the end of a tapered separation capillary or by connecting a metal-coated, full metal or conductive polymeric sprayer tip to the CE outlet. Another option is the insertion of microelectrode through the wall of the capillary end or to insert the electrode in a liquid junction. Although optimized sheathless set-ups [46-51] offer a strongly improved sensitivity, until now they have not been frequently used for CE-MS analysis of proteins, most likely due to their limited robustness, and lack of commercial availability. Moreover, this type of interface is only useful in a CE system which generates a significant EOF towards the outlet, as otherwise no effective spray is formed. It should be noted that CE systems with zero or low EOF, which often provide optimum resolution for complex protein mixtures, can be handled by the sheath-liquid interface giving it a key advantage in protein analysis.

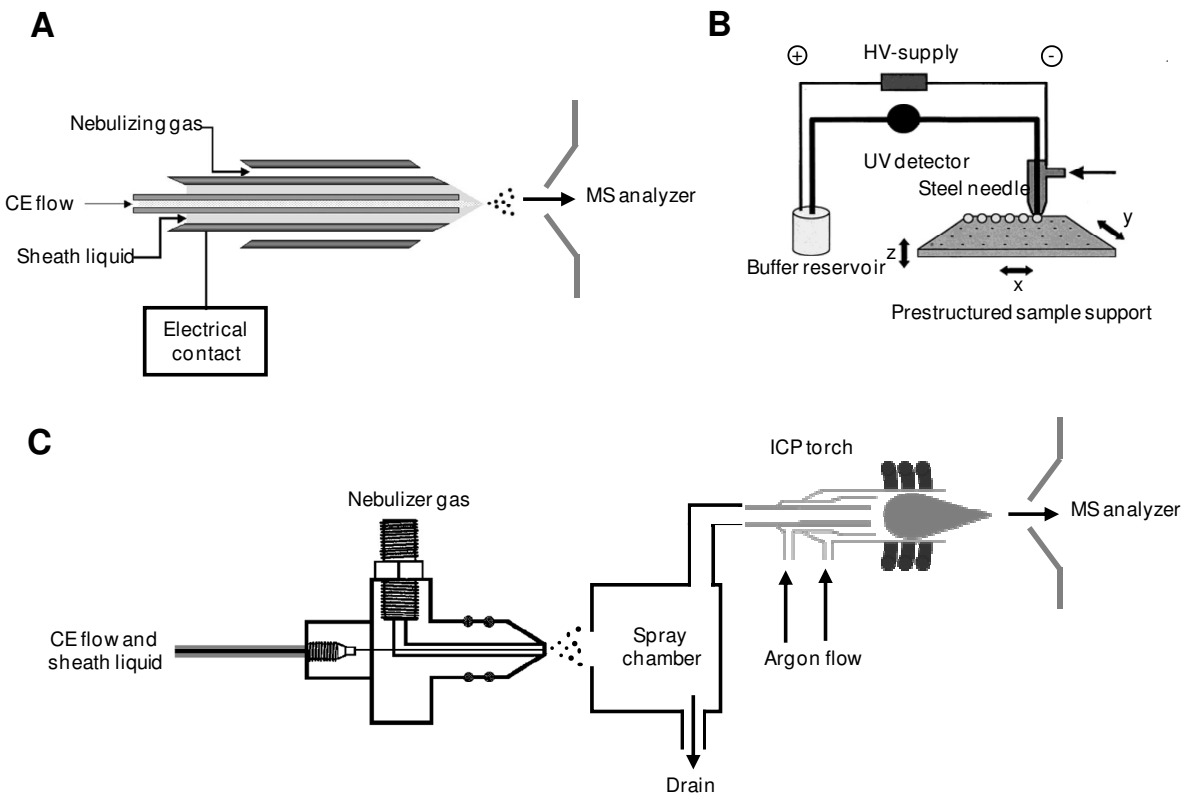

Figure 2.3. Sheath-liquid interfaces for (A) CE-ESI-MS, (B) CE-MALDI-MS [170] and (C) CE-ICP-MS.

The constituents of the BGE are an important aspect in CE-ESI-MS. Volatile buffers of low concentrations are preferred for sensitive ESI-MS, but these BGEs may not give optimum CZE separations. However, it has been shown repeatedly that satisfactory results can be obtained using BGEs of formic or acetic acid [17,23,52]. It should be noted that ammonia, often used to adjust the pH of the BGE, might negatively affect the ESI signal of analytes. It has also been shown that nonvolatile BGEs can be used for CZE-ESI-MS analysis of proteins. Phosphate buffers indeed result in a reduction of the MS intensity, but with borate buffers this effect is less severe and the obtained MS information can be adequate for exact mass determinations of intact proteins [53]. The addition of organic modifiers, like acetonitrile, to the BGE can improve the separation and MS detection performance of proteins [54], although care should be taken when noncovalent protein complexes are analyzed, as they could dissociate under the influence of the organic solvent [44]. In CIEF-ESI-MS the ampholytes that are required to create a pH gradient, may give rise to MS background signals and protein ionization suppression. The most common applied approach to deal with this, is to compromise between separation (favored by high ampholyte concentration) and detection (favored by low ampholyte concentration) performance [55-57]. Approaches that prevent the ampholytes from entering the ion source have also been developed. For example, microdialysis in principle may allow the removal of the ampholytes out of the CE effluent, allowing the interference-free detection of separated proteins [58,59]. However, this

somewhat complicated system has not been frequently applied for protein analysis due to the disturbance of the pH gradient by dialysis and the loss of protein resolution. The on-line combination of CIEF and reversed phase LC (RPLC) with ESI-MS detection is another option to circumvent ampholyte-related detection problems [60,61]. After focusing of proteins with CIEF, different p*I* fractions are transferred to a RPLC system, where the proteins are separated from the ampholytes prior to MS detection.

3.1.2 Matrix assisted laser-desorption ionization

MALDI-MS is widely applied for the analysis of intact proteins. For MALDI the sample and a UV absorbing matrix are mixed and the mixture is deposited onto a probe. With evaporation of the solvent, the analyte molecules co-crystallize with the matrix. After placing the probe into the vacuum of the MS, the protein spot is hit by a laser beam, which excites and partly vaporizes the matrix, bringing also the analyte molecules into the gas phase. In the so-called plume, protons are exchanged between analytes and matrix molecules, resulting in the formation of positively and negatively charged analyte molecules. From MALDI often just singly or doubly charged proteins will result, which makes the mass spectra relatively simple. Furthermore, MALDI is more tolerant than ESI to sample constituents like buffers and salts. In addition, MALDI-MS is able to measure proteins with masses up to hundreds of thousands of Daltons, which can be problematic with ESI. Unlike ESI, MALDI spectra give not as much information about proteins, as they merely provide their molecular mass. Although MALDI is more tolerable towards buffers and salt than ESI, adduct formation is very common with MALDI, which makes it more difficult to determine the exact mass of the protein.

It is difficult to perform on-line CE-MALDI-MS as MALDI usually has to be carried out in vacuum and the proteins have to be co-crystallized with the matrix on a surface before analysis. Therefore, off-line coupling systems were introduced, which allowed automated protein spotting [62,63]. The sheath-flow fraction collection interface has proven to be the most simple and straightforward approach to interface CE with MALDI (Figure 2.3B). A droplet of CE effluent is mixed with the matrix solution at the capillary tip and deposited on the MALDI target. After all fractions are collected on the MALDI plate, it is transferred into the mass spectrometer and analyzed. Various off-line interfaces have been proposed, mainly differing in the way the CE effluent is deposited on the MALDI plate [39,64]. For on-line coupling of CE and MALDI the rotating ball inlet [65] and vacuum deposition interfaces [66,67] were designed. With the on-line interfaces, the CE effluent is continuously deposited on a rotating object after it is mixed with a matrix solution. During the rotation the sample and matrix are dried and crystallized. The rotation places the matrix/analyte trace into the beam of the laser and the sample is introduced into the mass spectrometer. The on-line interfaces are relatively complex and have not been widely used for protein analysis.

3.1.3 Inductively-coupled plasma ionization

The application of ICP-MS in protein analysis mainly focuses on metal-containing or metal binding proteins. The sample is introduced as an aerosol into the heart of an argon plasma [68,69], operating at temperatures of 5000 – 9000 K. As the analytes pass the plasma they collide with free electrons, argon cations and neutral argon atoms, resulting in the complete breakdown of molecules into charged elements, usually in the M^+ state. Subsequently, in the mass spectrometer the specific elemental masses are monitored. Elements like carbon, oxygen and nitrogen are not favorable for protein detection as analyte signals are completely suppressed by the excess of these elements in, for example, the buffer or ampholytes used in CE. Therefore, ICP-MS is only useful for the analysis of proteins containing metals and/or other heavy atoms. It is important to note that ICP-MS does not give information on the molecular mass of a protein, but only about the presence of specific elements in the protein. In other words, separating proteins before introduction into the ICP torch is essential in order to distinguish different proteins in one sample.

In the basic design of a CE-ICP-MS interface, the capillary is placed in a nebulizer, through which the CE effluent is sprayed with the aid of a nebulizer gas (argon) into a spray chamber [70,71]. The droplets are than transferred to the ICP torch, where ionization can take place (Figure 2.3C). As with ESI-MS interfaces, the ICP interfaces use a sheath liquid in order to complete the electrical circuit for CE and to stabilize the formation of the aerosol [72]. The nebulizer is often self-aspirating and thereby creates a laminar flow in the capillary, reducing separation efficiencies. To prevent this, interfaces requiring low flow rates of sheath liquid were designed and tested for the analysis of intact proteins [71,73-80]. Other options to minimize band broadening by the interface are the use of long capillaries [81], the attachment of a short piece of small-diameter capillary to the end of the CE capillary [82] or the insertion of a frit into the inlet side of the CE capillary [83] in order to increase the flow resistance.

3.2 Mass analyzers

In order to obtain suitable mass spectrometric information from proteins, the choice of mass analyzer is important. Initially in CE-MS, quadrupole and ion trap (IT) mass analyzers were used in combination with ESI. These mass analyzers typically can cover a mass range of up to *m/z* 4000, allowing detection of multiple charged proteins as obtained from ESI. Disadvantage of the single quadrupoles is their relatively low resolution (about one mass unit) in the 100 – 2000 *m/z* range. This means that deconvolution of the mass spectra does not lead to a highly accurate mass of the protein and that it is impossible to distinguish between proteins which differ only a few mass units in molecular weight. IT analyzers show more favorable resolution at higher *m/z* ranges, however, the higher pressure and longer storage time results in an insufficient declustering making IT analyzers less suitable for intact protein analysis. Over the last decade, one can observe an increasing use of high resolution mass analyzers in CE-ESI-MS. Fourier-transform ion cyclotron resonance (FTICR) and time-of-

flight (TOF) mass analyzers provide the required high mass resolution and mass accuracy allowing unambiguous identification of protein species. For CE-MALDI-MS only TOF mass analyzers are used. As mass determination with these analyzers is based on ion flight time, the upper mass limit is virtually unlimited, making it highly suited for detection of singly charged high molecular weight compounds as resulting from MALDI. In CE-ICP-MS for proteins, quadrupole-based mass analyzers are still the most frequently employed. As the traces of specific elements have to be monitored, the mass resolution and accuracy of a quadrupole is sufficient.

4. Applications of CE-MS for intact protein analysis

4.1 Capillary zone electrophoresis - mass spectrometry

Quickly after Smith *et al*. showed that CE-MS coupling was possible with a sheath-liquid interface, they also demonstrated that proteins could be analyzed with CZE-ESI-MS [84]. Bovine insulin and two types of myoglobin were separated using CZE and molecular masses were determined with a quadrupole mass analyzer. Their research was the starting point for the exploration and application of CZE with MS detection for the analysis of intact proteins. An overview of protein CZE-MS is given below, making a subdivision in ESI, MALDI and ICP-based interfaces.

4.1.1 Electrospray ionization

CZE is the simplest mode of CE and ESI is very popular in protein MS. Not surprisingly, CZE-ESI-MS is the most frequently used in CE-MS of intact proteins. An overview of CZE-ESI-MS applications is given in Table 2.1 (p. 48), and as can be seen, they are divers and numerous. CZE-ESI-MS was first applied for the analysis of model proteins in order to develop and optimize the technique. Later its applications expanded towards more complex analytical problems like biomarker screening, isoform assignment and single cell analysis. Some interesting and typical examples, which illustrate the performance of CZE-ESI-MS, are treated in the following.

He *et al*. described the analysis of reduced and oxidized forms of cytochrome *c* by CZE-ESI-MS, using a sheath-liquid interface and an IT mass analyzer [85]. Cytochrome *c* containing Fe^{3+} can be reduced by thiol-containing compounds to the Fe^{2+}-containing protein. As a consequence, the proteins will have a charge difference of one and can be separated by CZE (Figure 2.4). The reduction was performed using the peptide Bcl-XL, and the separation was performed using a capillary coated with a bonded neutral polymer (no EOF). Using a BGE of pH 6 the positively charged proteins (p*I* 9.6) migrated towards the detector, whereas the excess of the negatively charged Bcl-XL peptide (p*I* 3.9) migrated towards the inlet of the capillary, and, therefore, did not interfere. The IT mass spectra did not show any difference between the reduced and oxidized cytochrome (Figure 2.4).

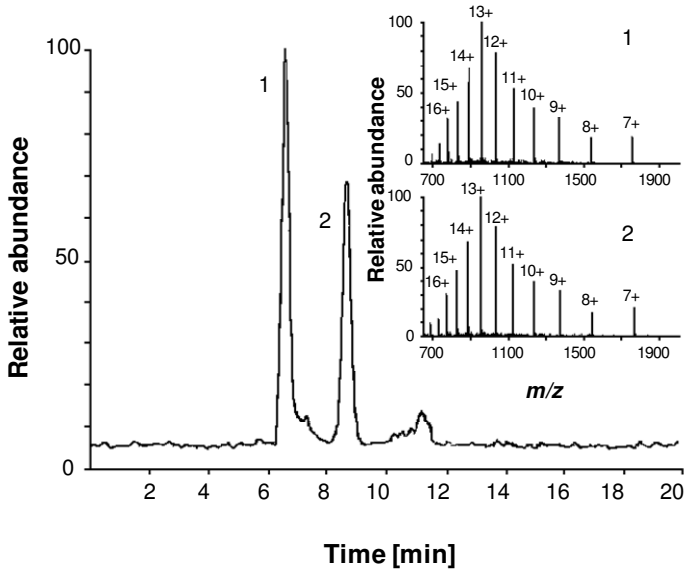

Figure 2.4. CZE-ESI-MS analysis of cytochrome *c* incubated with Bcl-XL peptide, showing both the oxidized (1) and reduced cytochrome *c* (2). Inserts show the ESI mass spectra of the peaks [85].

The combination of CZE with ESI-MS for protein and, especially, polypeptide analysis is frequently used in the field of biomarker discovery. Mischak *et al*. reported on several studies on the screening of various body fluids to identify possible biomarkers for certain diseases. Mainly urine was investigated [86-101], but also serum [99], plasma [102-104] and cerebrospinal fluid [89] were analyzed. The CZE-ESI-MS data were presented by plotting migration times of the observed constituents against their molecular masses, resulting in two-dimensional graphs. By comparing the graphs of diseased and healthy persons possible biomarkers were identified. A first publication was on the patterning of plasma dialysates obtained from two different dialyzer membrane types [104]. With a formic acid buffer of pH 2.5 containing 30% methanol as BGE, a sheath liquid of the same composition and a TOF mass analyzer, up to 2500 different polypeptides with masses above 1000 Da could be identified. In the next series of publications Mischak *et al*. investigated samples of patients suffering from renal diseases [89,90,92-99,101,102], ureamic toxicity [103], prostate [91] and urotelial cancer [87], ureteropelvic junction obstruction [88], sepsis and graft-versus-host disease [100]. In all cases known or possible new biomarkers were found using a method similar to the one described above. The CZE-ESI-MS method was compared with a surface enhanced laser-desorption ionization MS method. Only three possible biomarkers were found with the latter method and of these just one coincided with a compound found with CZE-ESI-MS [97]. CZE combined with MS is concluded to be very suitable for biomarker discovery.

Glycosylation is an important parameter in defining biological and biophysical properties of a broad range of proteins. Oligosaccharides are attached to the proteins posttranslationally and therewith glycosylation is a major source of protein heterogeneity. The carbohydrate structure plays an important role in determining biological activity and quite

some efforts have been made to characterize these proteins using CZE-ESI-MS [36,105-110]. The characterization of the glycoprotein EPO is a typical and intriguing example of this type of work [105-108]. In a recent study, Balaguer *et al*. showed the power of CZE in combination with high-resolution TOF-MS for glycoprotein analysis [105]. A commercially available polyacrylamide-based capillary coating in combination with an acetic acid BGE was used to separate the EPO glycoforms. From the obtained TOF-MS data and extracted ion electropherograms it could be concluded that the glycoform separation was mainly due to differences in amount of sialic acid residues, although differences in the hexose-N-acetyl-hexoseamine (HexHexNac) content also led to small differences in electrophoretic mobility, and thus partial separation (Figure 2.5A and 2.5B). Time windows in which comigrating isoforms of identical sialic acid content migrated were specified, and the mass spectra for that time period were averaged, to determine the presence of modifications like HexHexNac attachment, acetylation and oxidation (Figure 2.5C). The obtained mass information allowed the reliable distinguishment between the different EPO isoforms, however, unambiguous identification was not possible. Therefore, the glycans were released from the EPO using enzymatic deglycosylation and analyzed by CZE-ESI-MS/MS in the negative mode to determine the glycan identities. The overall information made it possible to fully characterize a European Pharmacopoeia reference preparation of recombinant human EPO. In total, 64 major glycoforms could be identified, and moreover, common glycan modifications as acetylation and oxidation were successfully characterized. This example clearly shows the power of CZE-ESI-TOF-MS, as this kind of information could not be obtained with other methodologies.

The analysis of metalloproteins can be performed with CZE-ICP-MS to obtain information about their metal content, as will be outlined in Section 4.1.3. However, CZE-ESI-MS can also be used to identify this type of proteins [111-116]. Metallothioneins (MTs) are proteins which are unusual in their structure, as they have a relatively low molecular weight and a high cysteine and metal content. The amount of different isoforms of MTs present in an organism is limited and a relatively simple CZE separation followed by a molecular weight determination by MS can be sufficient to identify these proteins in rat [112,114], rabbit [111,113,115], sheep [115] and baker's yeast [115] samples. Połeć Pawlak *et al*. showed that also metal displacement in MTs can be monitored with CZE-ESI-MS [116]. With the aid of bare fused-silica capillaries and a 30-mM ammonium acetate buffer (pH 7.2) the displacement of zinc by cadmium out of a heptameric zinc-metallothionein complex from mice was measured after the gradual addition of cadmium. The stochiometry of the metal displacement could be established and important evidence for the structural importance of the last remaining zinc ion was obtained.

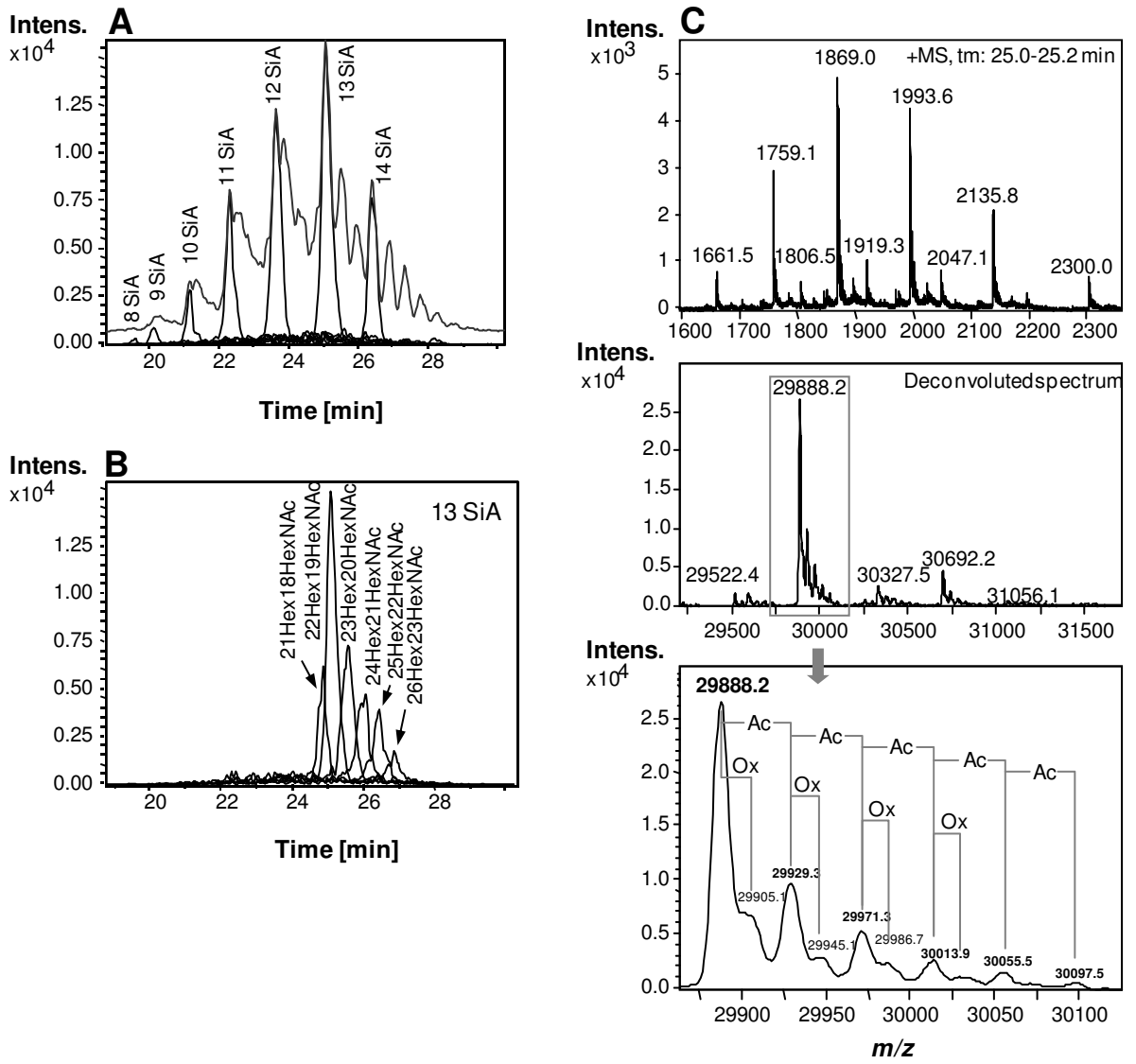

Figure 2.5. CZE-ESI-MS analysis of a European Pharmacopoeia reference preparation recombinant human EPO showing (A) different sialic acid (SiA) isoforms, (B) different HexHexNac content of one specific SiA isoform, and (C) the original mass spectrum and the deconvoluted spectrum of one specific isoform, revealing acetylated (Ac) and oxidized (Ox) isoforms [107].

Other clinically relevant metal-binding proteins have also been examined by CZE-ESI-MS. For example, the analysis of hemoglobin [117-120] and carbonic anhydrase [117] in human red blood cells has been reported. Aminopropylsilane coated capillaries were used in combination with acetic acid BGEs in order to minimize interactions with the capillary wall and to create a stable EOF. Hofstadler *et al*. described the analysis of hemoglobin in a single red blood cell (~90 fL) with CE and high resolution mass spectrometry [119]. An FTICR mass analyzer was used in combination with a 20-µm ID CE capillary and the α- and β-chain of hemoglobin were detected in the cell lysate by scanning a small mass range to improve sensitivity. Cao and Moini used a similar methodology, with a 30-µm ID capillary in combination with a TOF mass analyzer [118]. No baseline separation of the cellular content

was obtained, but monitoring of specific mass traces allowed detection of the α- and β-chain of hemoglobin.

The analysis of carbonic anhydrase I and II in red blood cells is rather challenging due to the relatively low concentration, which is 70 and 700 times lower compared to hemoglobin, respectively. Moini showed the CZE-ESI-MS analysis of both hemoglobin and carbonic anhydrase [117]. The α- and β-chain of hemoglobin and carbonic anhydrase I could be separated and detected, however, the carbonic anhydrase II concentration was below the detection limit. Although the dynamic range was limited, it was clearly shown that CZE-ESI-MS can be used for the determination of specific proteins in complex samples.

As protein concentrations may be low, Stutz *et al*. developed a CZE-ESI-MS method in which different clinically relevant metal-binding proteins could be preconcentrated using an isotachophoresis step [27]. Using a formic acid BGE, five different metal-binding proteins could be separated and identified with limits of detection down to sub-femtomole per liter. This system looks promising, but until now has not yet been applied to real samples.

Above described examples deal with the analysis of target proteins in a sample. Moini and Huang described the unbiased analysis of intact ribosomal proteins from the K-12 strain of *Escherichia coli* (*E. coli*) using CZE-ESI-MS [121]. By performing two separate runs with

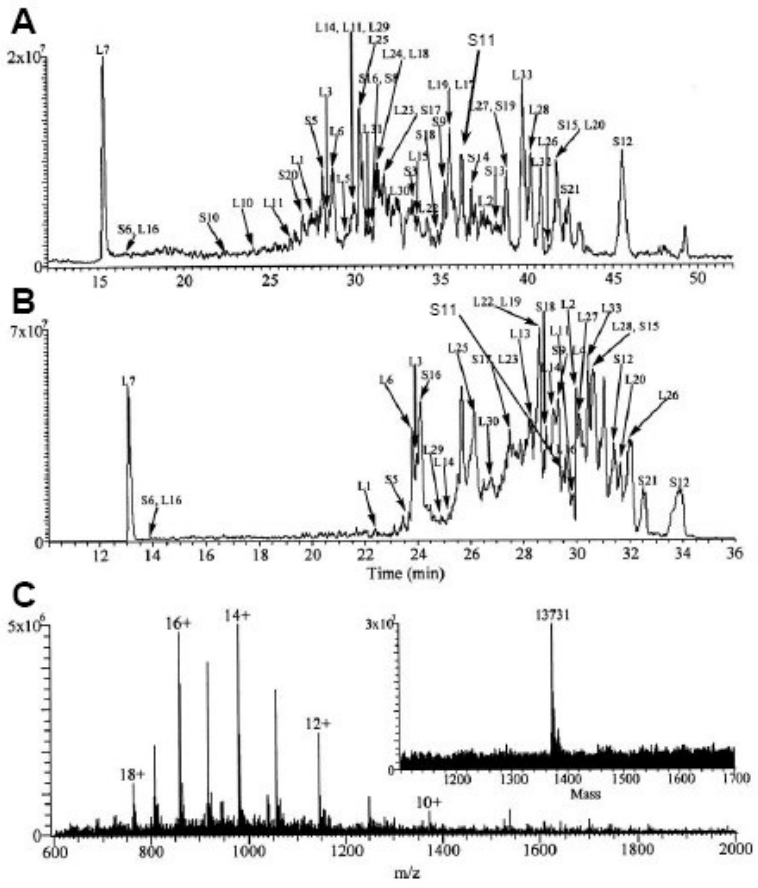

Figure 2.6. CZE-ESI-MS analysis of ribosomal proteins from E. coli using a BGE consisting of (A) 0.1% acetic acid in 50% acetonitrile and (B) 0.1% acetic acid in water. (C) Measured and deconvoluted spectrum of peak S11 [121].

either a 0.1% acetic acid BGE or a BGE consisting of 0.1% acetic acid in 50% acetonitrile, both in combination with a aminopropylsilane-coated capillary and an IT mass analyzer, 55 proteins out of 56 expected could be identified (Figure 2.6). Addition of acetonitrile to the BGE improved the separation of certain proteins, which were not separable with the aqueous BGE. Proteins were assigned by deconvolution of the obtained mass spectra of the separated peaks and comparing these masses with literature values. The high identification grade was possible as most proteins were resolved by CZE and present in the same concentration range.

Cifuentes *et al*. determined phycobiliproteins from blue-green algae or *Spirulina platensis* using CZE-ESI-MS [122,123]. These proteins are relatively difficult to extract from the organism as it has a very rugged cell wall. Therefore, a pressurized liquid extraction method was used to isolate the proteins from the algae. Bare fused-silica capillaries with thorough rinsing steps between runs and a BGE containing 40 mM ammonium hydrogen carbonate (pH 7.8), 50% acetonitrile and 5% isopropanol allowed for reproducible CZE-ESI-MS analyses. Four major phycobiliproteins could be separated and identified based on their measured molecular weight.

4.1.2 Matrix assisted laser-desorption ionization

The initial publications on CZE-MALDI-MS all deal with setting up appropriate interfacing using model proteins. Keough *et al*., used CZE to separate lactalbumin, myoglobin and carbonic anhydrase after which the protein zones were manually collected and spotted on a MALDI target plate. The MALDI-MS analysis proved that one of the peaks contained both myoglobin and carbonic anhydrase [124]. Castoro *et al*. introduced Fourier Transform MS for CZE-MALDI-MS [63]. Somastatin, myoglobin and insulin were separated with CZE and fractions were deposited on the MALDI plate using a sheath-liquid MALDI interface. The Fourier Transform MS analysis confirmed the identity of the proteins in the three deposited fractions. Based on these two publications, other authors also tried to combine CZE with MALDI-MS for protein analysis. However, as can be seen in Table 2.2 (p. 53), the number of publications on CZE-MALDI-MS for intact proteins is limited.

A typical example of data obtained with CZE-MALDI-MS is given in Figure 2.7. Zuberovic *et al*. separated six model proteins and spotted the CE effluent on a MALDI plate [125]. A physically adsorbed cationic PolyE-323 capillary coating was used to obtain good protein separations. Spotting was performed with a sheath flow addition of matrix on the MALDI target plate. MALDI spectra of the pure proteins could be obtained showing that the CZE separation was essentially maintained during the spotting procedure. This CZE-MALDI-MS system was used for the analysis of tear fluid in which lysozyme and lactoferrin could be identified.

Chakel *et al*. used CZE-MALDI-MS for the analysis of glycoproteins to show that it is a suitable technique to determine consistency in recombinant techniques [126]. With bovine serum albumin treated capillaries and a sodium phosphate buffer (pH 3.0) Desmodus salivary

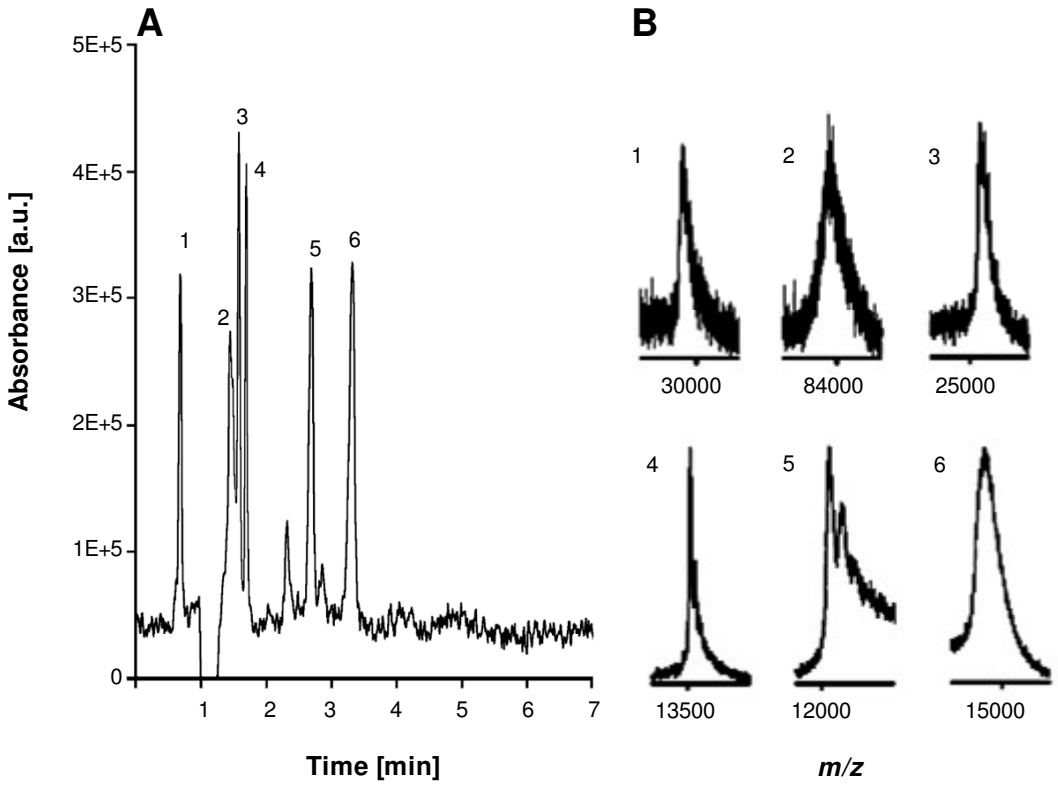

Figure 2.7. (A) CZE-UV analysis of a protein mixture containing carbonic anhydrase (1), lactoferrin (2), α-chymotrypsinogen A (3), ribonuclease A (4), cytochrome *c* (5) and lysozyme (6). (B) MALDI-MS spectra of the proteins separated by CZE [125].

plasminogen activator glycoforms could be partially separated. MALDI-MS analysis of the proteins present in four deposited fractions showed that still more than one glycoform was present in each peak. Better separation would be necessary to fully characterize the protein and its glycoforms.

Cachexia, or muscle loss, decreases the survival rate of cancer patients. A sulfated glycoprotein was found to be associated with this muscle breakdown. Therefore, Choudhary *et al*. analyzed urine samples from healthy and diseased persons in order to screen for this protein [127]. After CZE separation, fraction collection and an additional CZE separation, MALDI-MS analysis of deposited fractions from the last CZE separation revealed, next to albumin, the glycoprotein associated with cachexia. As the peak in the mass spectrum was broad, it was suggested to consist of multiple glycoforms.

4.1.3 Inductively coupled plasma ionization

CZE-ICP-MS is limited to metal-containing and metal-binding proteins. However, for this type of proteins highly selective information can be obtained. Table 2.3 (p. 54) summarizes the applications of CZE-ICP-MS. Metallothioneins (MTs) are the most frequently analyzed proteins with CZE-ICP-MS. The analysis of MTs is very straightforward, as in most cases a bare fused-silica capillary with a buffer around physiological pH (6.8-7.8)

gives sufficient separation [24,26,71,74-76,78-80,83,128-134]. The identity of the MTs is determined by analyzing the traces of copper, zinc, cadmium and/or sulfur. This can provide stoichiometric information on the proteins if more than one element is monitored. MTs have been analyzed by CZE-ICP-MS in a variety of matrices including human brain cytosols [133], mussel hepatopancreas cytosols [134], rabbit liver [24,131,135], eel liver [26], rat liver [112,132], roe deer liver [129], breem liver [129,130] and cyanobacterium Synechococcus [136]. Due to the element-specific detection, matrix components do not interfere with ICP-MS, which makes the analyses rather straightforward.

Wang *et al*. used a covalent 2-acrylamido-2-methyl-1-propanesulfonic acid coating in combination with TRIS (pH 7.4) as BGE for CZE-ICP-MS [24]. Nine different MTs could be resolved in a rabbit liver sample, while monitoring the traces of sulfur, copper, zinc and cadmium allowed for the stoichiometry of the different MT isoforms to be estimated (Figure 2.8). The MTs, coming from a commercial rabbit liver MT solution, were found to be isoforms of MT1 and MT2 and one unknown MT. As ICP and ESI give complementary information, CZE-ICP-MS and CZE-ESI-MS have been used together for MT analysis in order to unambiguously determine which isoforms are present in a sample [112,132,135].

Chamoun *et al*. applied CZE-ICP-MS in combination with LVSS for determination of metal-binding properties of carbonic anhydrase, bovine serum albumin, human holotransferrin, ceruloplasmin and superoxide dismutase [25]. The proteins were incubated with Cd^{2+} and subsequently separated by CZE using a high-pH BGE. LVSS was used, as the

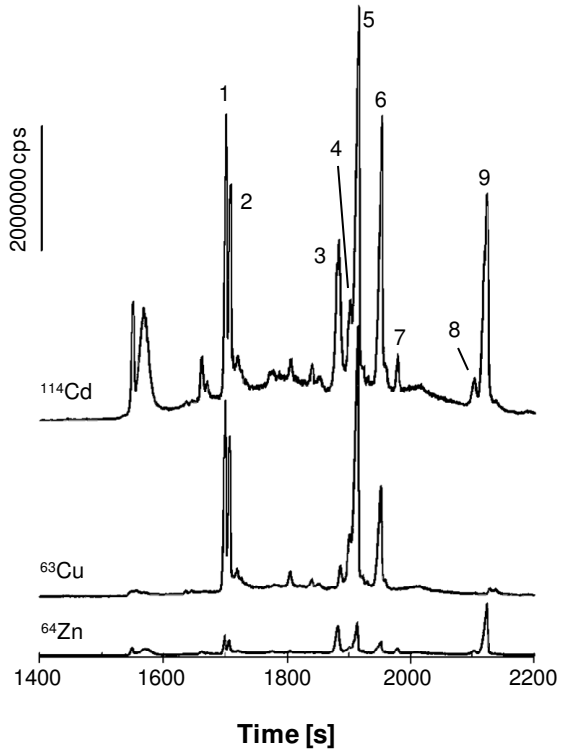

Figure 2.8. CZE-ICP-MS analysis of a rabbit liver MT sample using a 2-acrylamido-2-methyl-1-propanesulfonic acid coated capillary. The proteins are detected by their cadmium, copper and zinc content as monitored with ICP-MS. Numbers indicate the MT isoforms which were identified [24].

interactions between metals and these proteins are weak and low concentrations of metal-protein complex were expected. With this method, the interaction of Cd^{2+} with carbonic anhydrase, bovine serum albumin and ceruloplasmin was proven. By using ICP-MS the interferences from excessively present non-interacting proteins could be circumvented.

Michalke described the manganese speciation in liver extracts with CZE-ICP-MS [137]. It is known that manganese in the liver can bind to different compounds, including proteins. By the CZE-ICP-MS analysis of low-molecular weight compounds and protein standard solutions incubated with manganese, the binding of manganese to these compounds could be demonstrated. Subsequently, the analysis of liver extracts was performed and manganese-bound transferrin and albumin could be identified at µg/kg levels.

Timberbaev *et al*. showed that CZE-ICP-MS is an attractive tool for metallodrug-protein binding studies. CZE-ICP-MS conditions to monitor the binding of platinum-containing compounds to human serum albumin were optimized [138]. Subsequently, the reactivity of an antitumor ruthenium(III) complex towards human serum proteins, like albumin and transferrin, was investigated [139]. After optimization of the set-up and separating the bound and non-bound fractions, binding constants towards both proteins were determined. It was shown that the antitumor agent has more affinity towards albumin compared to trasferrin. It was concluded that binding properties found with CZE-ICP-MS were in agreement with in vivo experiments.

4.2 Capillary isoelectric focusing - mass spectrometry

In 1995, Tang *et al*. showed the feasibility of coupling CIEF with ESI-MS using model proteins [57]. The same year Foret *et al*. showed MALDI-MS of model proteins separated by CIEF [140]. These publications triggered other researchers to study CIEF-MS and today quite some papers have appeared in this field, especially using ESI for interfacing. So far, CIEF has not been coupled to ICP-MS for intact protein analysis.

4.2.1 Electrospray ionization

CIEF-ESI-MS first found its use in the analysis of model proteins and then developed into a suitable method for the characterization of hemoglobin variants and proteome analysis. Table 2.4 (p. 56) summarizes the applications of CIEF-ESI-MS.

One of the major potential problems in on-line CIEF-ESI-MS is the presence of the ampholytes. Upon mobilization of the separated protein zones, ampholytes may flow into the ion source of the mass spectrometer where they can cause ionization suppression of the protein analytes. The effect of the ampholytes in CIEF-ESI-MS has been studied in more detail by Tang *et al*. [57]. It was shown that with increasing ampholyte concentrations protein zone widths narrowed and separation efficiencies improved, whereas the protein MS signals decreased rapidly. For sufficient separation an ampholyte concentration of at least 0.5% appeared to be required. At this ampholyte concentration, the MS signal intensities were about

four times lower as compared to the situation in which no ampholytes are present. These findings were generally confirmed by others [55,56,141] and regarded as acceptable. Currently, in most CIEF-ESI-MS applications ampholyte concentrations of 0.5 to 1.0% are used, compromising between separation efficiency and signal intensity. Methods to remove the ampholytes prior to MS detection are presently hardly applied as they often are too complex and/or sacrifice too much of the protein resolution obtained by CIEF.

Martinovic *et al*. showed the separation of noncovalent protein complexes with CIEF and subsequent detection via ESI-MS [142]. The separation of protein complexes GADH and CPK was accomplished with a neutral polyacrylamide coated capillary containing a pH gradient from 3 to 10. After mobilization, the acidic sheath liquid and high voltages of the electrospray interface caused dissociation of the complexes. This resulted in the detection of monomeric species using a FTICR mass analyzer (Figure 2.9A). By using a medium-pH sheath liquid and lower ESI voltages complexes could be preserved (Figure 2.9B) and from the molecular masses stochiometry could be determined.

Jensen *et al*. showed CIEF-ESI-MS as a tool for monitoring protein refolding induced by disulfide bond formation [143]. The in vitro folding of ribonuclease A was blocked at different stages by alkylating free thiols with iodoacete. The reaction of free thiols with iodoacetate added one negative charge for each alkylation site, resulting in the decrease of p*I* and an increase of migration time of refolding intermediates. The refolding intermediates were identified on the basis of the mass spectra taken from the average of scans under the

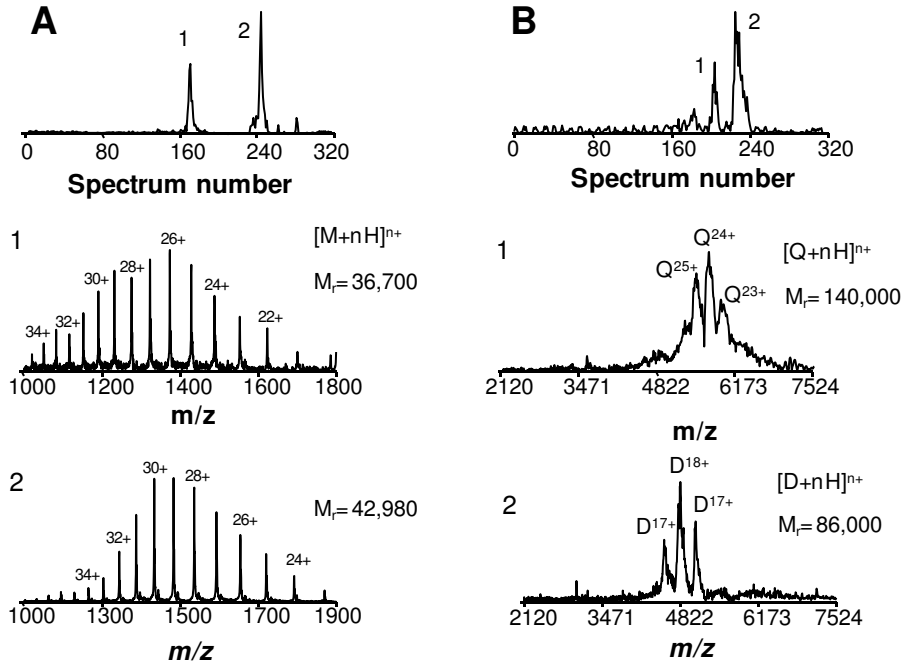

Figure 2.9. CIEF-ESI-MS analysis of a mixture of the noncovalent protein complexes GADH (1) and CPK (2). (A) Mass spectra of monomeric, dissociated species are obtained upon addition of an acidic sheath flow and high ESI voltages. (B) Mass spectra of GAPDH tetrameric and CPK dimeric complexes are obtained with a medium-pH sheath liquid and lower ESI voltages [142].

peaks. With increasing refolding, the mass of the protein decreased, as less iodoacetate could react with the free thiols. From the combination of differences in molecular mass and the reduction in p*I* of alkylated folding intermediates, obtained with CIEF-ESI-MS, the refolding pathway was monitored and the refolding kinetics were studied.

Tang *et al*. compared CIEF with CZE for the analysis of model proteins using ESI-MS for detection [144]. In both cases the EOF was suppressed by using a neutral polyacrylamide capillary coating. In the CZE experiments, a 20 mM ε-aminocaproic acid buffer (pH 4.4) was used to ensure that all proteins were positively charged and migrated towards the detector. For the CIEF analysis, a pH gradient from 3 to 10 was used in a one-step approach, in which the proteins were focused during mobilization. Loss of resolution due to moving ionic boundaries was circumvented in CZE by using a common counterion in the BGE and sheath liquid. With CIEF-ESI-MS, concentration limits of detection were at the sub-μM level, which was approximately 20 to 50 times lower than with CZE-ESI-MS. It was shown that both with CIEF and CZE no full protein separation could be obtained, but that the techniques demonstrated a different selectivity. For example, with CIEF two myoglobin variants could be partially separated, while CZE allowed the separation of β-lactoglobulin A and carbonic anhydrase I. Therefore, it can be concluded that both CZE-ESI-MS as CIEF-ESI-MS give complementary information for the analysis of complex samples.

The analysis of hemoglobin variants is often hindered by the fact that the variants are very similar and, therefore, difficult to separate. With CIEF it is possible to separate hemoglobin variants, although their p*I* differs only 0.05 p*I* units [141,145-147]. Tang *et al*. separated hemoglobin variants A, C, F and S after focusing in a polyacrylamide-coated capillary containing a pH gradient from 5 to 8 [141]. Hemoglobin consists out of four noncovalently bound chains: 2 α-chains, which are present in each variant, and 2 β- or γ-chains, which differ per variant. In the ESI interface hemoglobin can dissociate and by scanning specific single mass traces, corresponding to the different chains, the hemoglobin variant can be identified (Figure 2.10). Concentration limits of detections were calculated to be in the range of 10^{-8} M and were attributed to the focusing during mobilization and the selective detection of the hemoglobin chains, which minimizes interferences of other constituents.

Clarke and Naylor showed that is possible to determine hemoglobin in whole human blood with CIEF-ESI-MS without any sample preparation [145]. One of the major problems that had to be circumvented was the presence of high concentrations of physiological salts. A voltage gradient was applied before focusing the proteins in order to migrate the salts out of the capillary [145,148]. The electropherograms were still quite complex with overlapping components, but the high concentration of hemoglobin and the selection of specific mass traces for the hemoglobin chains made it easy to analyze the protein. Blood samples from healthy and diabetic persons were compared and the deconvoluted mass spectra obtained from

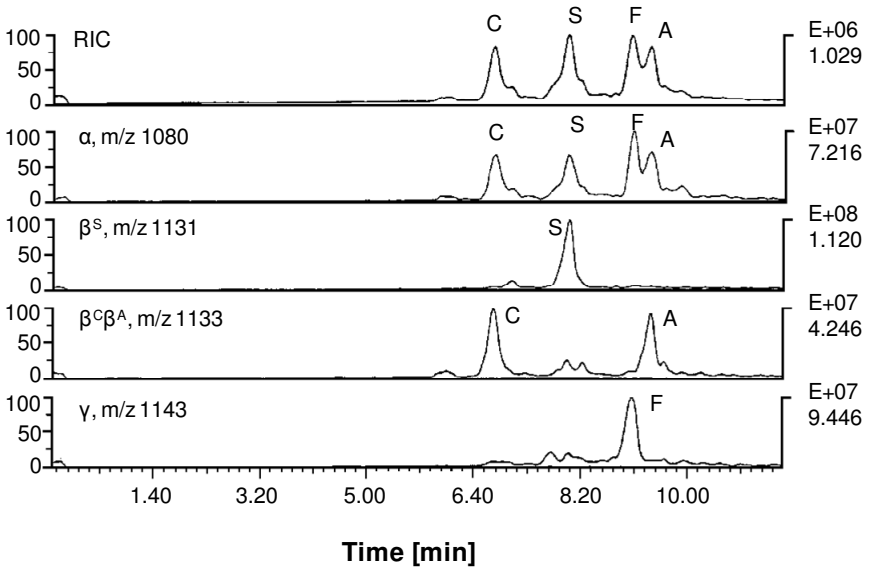

Figure 2.10. CIEF-ESI-MS analysis of hemoglobin variants A, C, F and S. Mass traces belonging to the different specific hemoglobin chains (α, β and γ) were monitored in order to identify the hemoglobin variants. The upper trace represents the reconstructed multiple-ion electropherogram [141].

the hemoglobin peak showed an increase in glycated α-chains in diabetic persons. This was somewhat surprising as it was assumed that only the β-chain is glycated.

Smith *et al*. reported on proteome analyses with CIEF-ESI-MS and an FTICR mass analyzer [149-155]. In all cases E. coli samples were used and the emphasis of the research was to improve the analysis of complex samples with CIEF-ESI-FTICR-MS. With aid of the high resolution power of ESI-FTICR-MS multiple overlapping charge envelopes could be discriminated [149]. Furthermore, the selective incorporation of isotopically labeled amino acids [150,152], application of isotope depleted medium [153-155] and stepwise mobilization of the focused proteins [151] was described. Using the selective incorporation of isotopically labeled amino acids allowed a more accurate identification as the molecular mass of a protein could be combined with the number of one specific amino acid. The isotopically depleted medium was used to increase mass accuracy as isotopic masses no longer coincide, whereas the stepwise mobilization allowed for an increase in resolution between proteins and a more controlled release of the proteins into the mass spectrometer. In total 400 to 1000 possible proteins were found applying these latter methods in combination with CIEF-ESI-FTICR-MS.

Zhou and Johnston report on the application of the two-dimensional approach, using CIEF and RPLC combined with ESI-MS for the analysis of intact proteins [60,61]. In this hyphenated system comigrating proteins from the same CIEF p*I* fraction are separated before ESI-MS detection. This method was applied to a yeast sample, detecting up to 500 possible proteins and polypeptides in the mass range from 3 to 30 kDa and with a p*I* between 3 and 11. The same approach was used by Wang *et al*., who report a similar amount of proteins in a yeast sample, with the difference that the mass range was larger, going up to 70 kDa and their p*I* range was limited between 3.3 and 9.6 [15].

4.2.2 Matrix assisted laser-desorption ionization

CIEF-MALDI-MS is the least developed CE-MS method for the analysis of intact proteins. Up to now its use is limited to basic research, analyzing model proteins in order to develop and optimize the technique. In Table 2.5 (p.58) these applications are listed. MALDI is less sensitive towards matrix components than ESI and, therefore, often higher ampholyte concentrations are applied in CIEF-MALDI-MS, as can be seen in the table.

Foret *et al*. [140] studied the CIEF-MALDI-MS analysis of the model proteins myoglobin, carbonic anhydrase I and II and β-lactoglobulin A. Neutrally coated capillaries were used to minimize the EOF applying a pH range from 3 to 10 and a sample was collected every 65 seconds with a sheath-liquid interface to make manual droplet collection possible. The four proteins were efficiently separated and MALDI spectra showed masses that were in agreement with literature. Next, a sample containing several hemoglobin variants with small differences in p*I* was analyzed. As the resulting peaks were more closely spaced the fraction collection speed was increased and fractions were collected every 10 seconds. The separation was nicely maintained upon deposition, however, as the variants all had similar molecular masses they could not be distinguished by MALDI-MS.

Minarik *et al*. [156] and Chartogne *et al*. [157] both focused their research in CIEF-MALDI-MS on fraction collection. Minarik used two UV detectors in series to determine accurate zone exit time and collected the proteins fractions manually. Furthermore, 200 μm ID polyvinylalcohol-coated capillaries were applied to increase the injected amount, a semipermeable membrane was used to prevent bulk flow and a slowly increasing focusing voltage was necessary to reduce Joule heating. Myoglobin, carbonic anhydrase II, β-lactoglobulin and ovalbumin were separated, collected in tubes and spotted manually on the MALDI plate. It was shown that the fraction collection speed influenced the separation, while MALDI-MS experiments confirmed the identity of the proteins. Chartogne *et al*. did not first collect fractions before MALDI-MS analysis, but directly spotted the sample on the MALDI plate using a sheath liquid and adding the matrix afterwards manually. After optimization four model proteins were separated and good quality MALDI spectra were obtained.

Crowley was the first to analyze real samples with CIEF-MALDI-MS [158]. A CIEF separation with a suppressed EOF was performed and protein bands were spotted on a MALDI plate with a sheath flow interface, while the matrix was afterwards added manually. After the removal of albumin and immunoglobin G from serum samples, proteins with masses up to 100 kDa were detected (Figure 2.11). The proteins could not be identified unambiguously, but CIEF-MALDI-MS was found to be advantageous over 2-D gel electrophoresis as the entire mass range from 2 kDa to 200 kDa could be analyzed in a single run. Furthermore, in 2-D gel electrophoresis, information on proteins with masses lower than 10 to 30 kDa may be lost, which was not the case in this CIEF-MALDI-MS application.

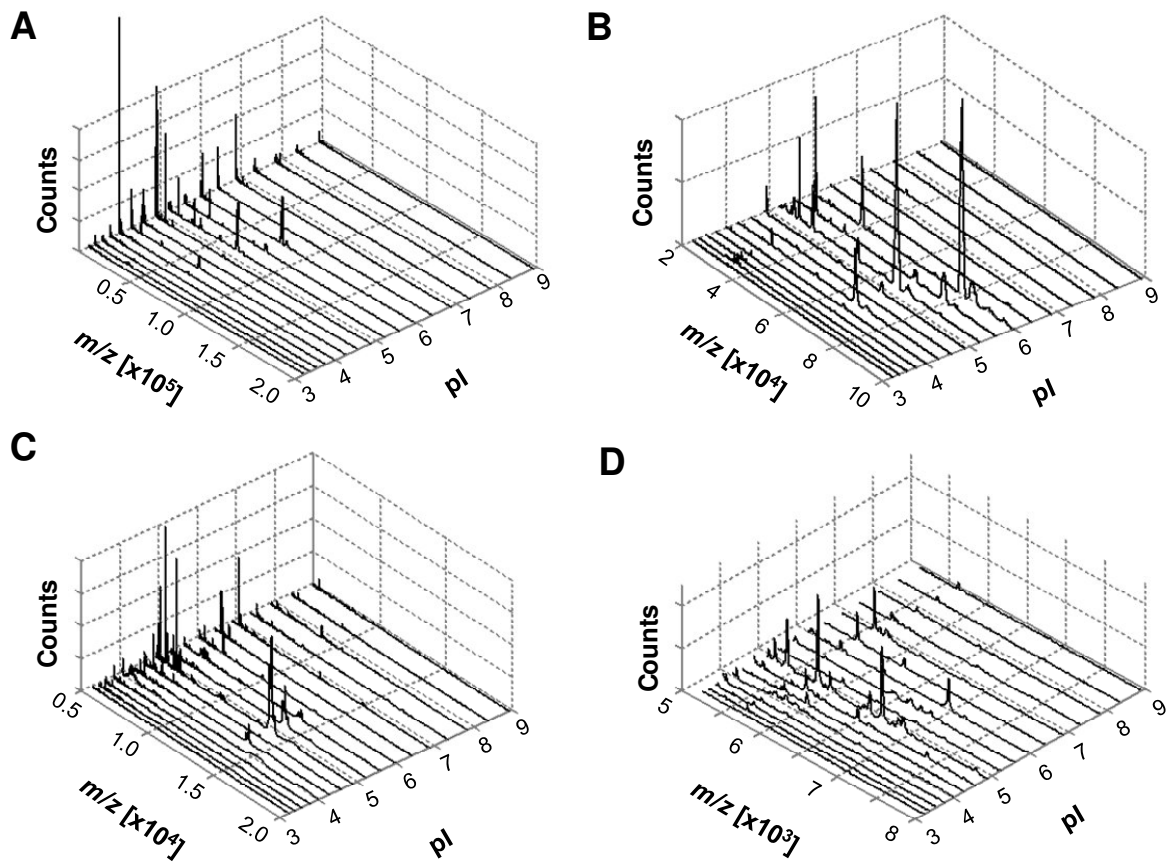

Figure 2.11. CIEF-MALDI-TOF-MS analysis of a human serum sample, showing (A) the full *m/z* range and the mass ranges (B) 20 - 100 kDa, (C) 5 - 20 kDa and (D) 4 - 8 kDa [158].

4.3 Chip-based electrophoresis combined with mass spectrometry

Recently, there has been an increased interest in the development of chip-based analytical systems, as they may increase analysis speed and performance, and reduce cost, weight and size of the instrumentation. Successful efforts have been made to combine microfluidic CE systems with mass spectrometric detection [159,160]. In the protein analysis field, applications of chip-based CE-MS mainly focus on digests of proteins, but intact protein analysis has also been described in some cases. The combination of on-chip CE combined with MS for intact proteins is performed with either CZE or CIEF and with ESI-MS or MALDI-MS (Table 2.6; p. 58). In these applications chip materials like glass [161,162], quartz [163], polycarbonate [164], polymethyl methacrylate [165] and polyester [166] are employed, sometimes in combination with a capillary coating in order to minimize protein adsorption [161].

4.3.1 Capillary zone electrophoresis

Miniaturized systems employing a voltage to introduce a sample into the mass spectrometer are frequently described in literature [167-169]. However, in these instances no separations are performed as the device is merely used for direct infusion of a sample into the

mass spectrometer. Currently, few examples of chip-based CZE separations before mass spectrometric detection of intact proteins are found in literature. Zhang *et al*. showed the CZE separation of intact model proteins in combination with ESI-MS detection [162]. A liquid junction interface was employed in combination with an 11 cm long etched separation channel (Figure 2.12A) and a low-pH buffer for the separation of acidic, neutral and basic proteins. To prevent adsorption of the proteins polyacrylamide and polyvinyl alcohol coatings were applied. In four minutes cytochrome *c*, myoglobin, β-lactoglobulin A and B were baseline-separated and the obtained mass spectra confirmed the identity of the proteins (Figure 2.12B).

The analysis of basic proteins has also been described by Akashi *et al*., applying the polymer wall coating polyE-323 [163]. Ten repetitive injections at intervals of one minute showed the theoretical separation of lysozyme, myoglobin and the DNA-binding domain of human TTAGGG repeat binding factor 2 (hTRF2 DBD) in a 6 cm long channel within 30 seconds. Deconvoluted mass spectra confirmed the identity of the proteins. After these initial experiments, repetitively injected hTRF2 DBD was analyzed at different mass spectrometric

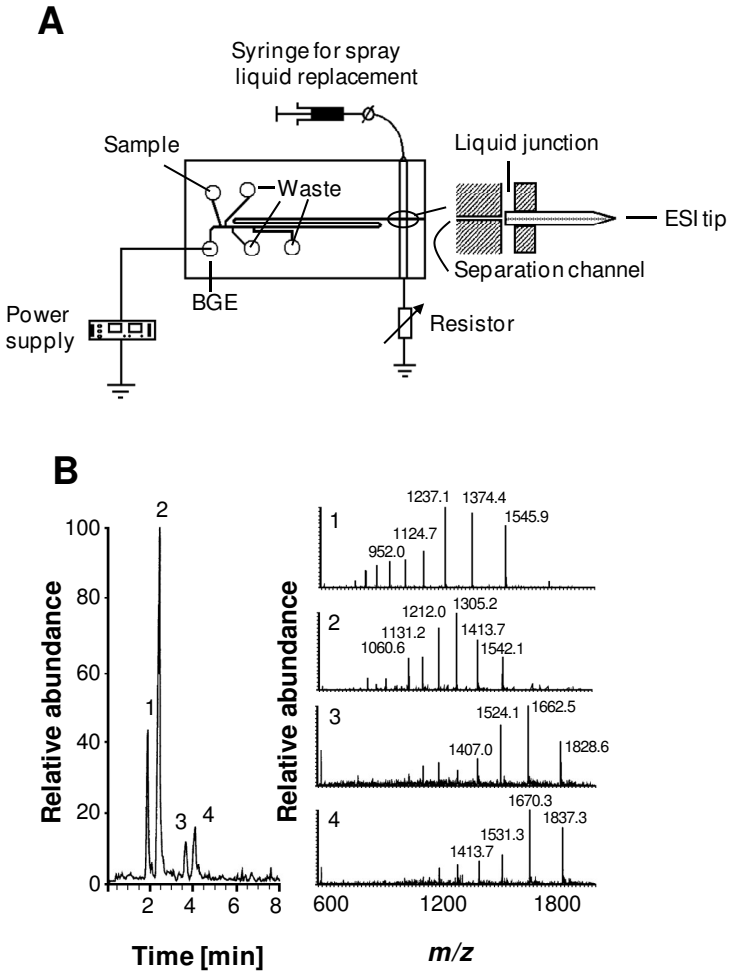

Figure 2.12. (A) Schematic of a microdevice applied for the CZE-ESI-MS analysis of intact proteins. (B) Chip-based CZE-ESI-MS analysis of a mixture of cytochrome *c* (1), myoglobin (2), β-lactoglobulin B (3) and β-lactoglobulin A (4), showing the base peak electropherogram and the mass spectra of each peak [162].

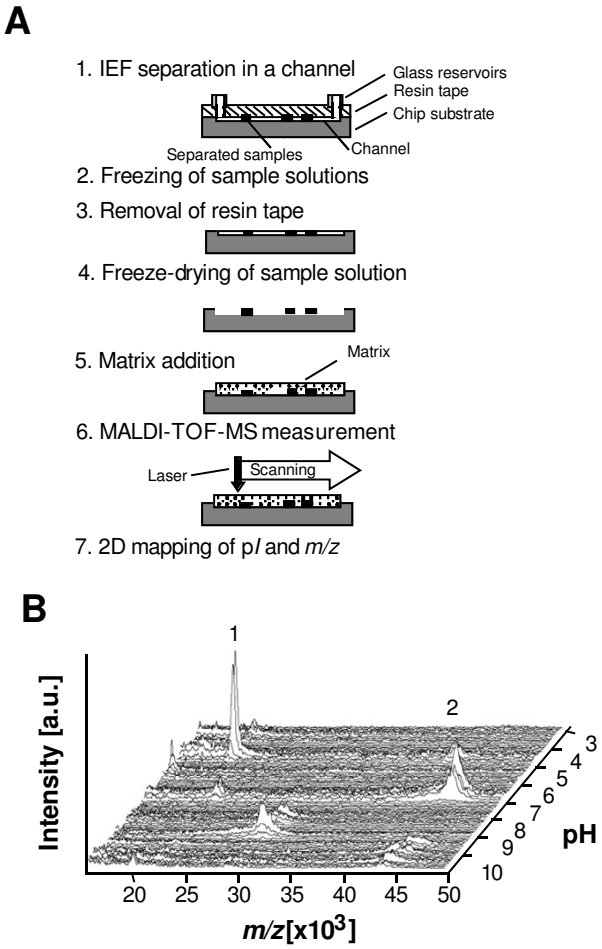

Figure 2.13. (A) Schematic representation of the experimental procedure to acquire a 2-D graph with chip-based CIEF-MALDI-MS. (B) 2-D graph of p*I* vs. *m/z*, as shown for trypsin inhibitor (1) and creatine phosphokinase (2) [161].

settings. During these analyses fragments from the intact protein were found, suggesting that identification by fragmentation of intact proteins after a chip-based separation is possible.

4.3.2 Capillary isoelectric focusing

Fujita *et al*. described the chip-based CIEF-MALDI-MS analysis of creatine phosphokinase and trypsin inhibitor. After focusing the proteins in the separation channel on the chip, which is covered with a resin tape, the channel content is frozen, the tape is removed and the sample solution is freeze-dried to prevent migration of the solution and maintain resolution. After this procedure the matrix is added to the chip (Figure 2.13) [161]. . Scanning with the MALDI laser across the surface gave information about the p*I* (position of chip) and molecular weight (mass spectrum). The sample zones did not deteriorate during the freeze-drying process, as was measured using a fluorescence detector after staining the proteins with a fluorescent cyanide dye.

Sze *et al*. performed chip-based CIEF-MALDI-MS with a polymethyl methacrylate chip for the analysis of myoglobin, carbonic anhydrase and trypsin inhibitor [165]. After

focusing the proteins in a seven cm long channel, the matrix was added to the chip and the laser was used to scan across the surface of the chip in order to detect the proteins. In a subsequent publication, the fabrication process and the material of the chip were optimized, giving higher detection sensitivity compared to the previously used chips [166]. With a polyester chip the CIEF separation of five model proteins ranging from acidic to basic was performed. The molecular masses were determined with MALDI –MS by scanning the laser across the chip surface.

Wen *et al*. showed the analysis of three model proteins with CIEF-ESI-MS on a chip by coupling via a sheath-liquid interface [164]. Using a 16 cm long separation channel without any coating, they optimized the flow rate of the sheath liquid and nebulizing gas in order to gain a stable spray. A separation of carbonic anhydrase and myoglobin was accomplished and the obtained mass spectra allowed the identification of the proteins. The focusing and mobilization was established within five minutes and although the separation was not as efficient as with normal CIEF-ESI-MS, this design showed the feasibility of chip-based CIEF-ESI-MS.

5. Conclusion

Over the last years, one can observe a growing demand for separation methodologies that allow characterization of intact proteins. This is especially true in the pharmaceutical environment, where an increasing number of drugs are proteins. Researchers in academia and companies start to recognize some of the advantages of CE over LC in this area. As MS is an increasingly important tool for intact protein analysis and CE-MS coupling techniques are available, it is not remarkable that the number of publications in the field of CE-MS of intact proteins has expanded steadily. In the initial stage, studies were merely focused on demonstrating the feasibility of intact protein analysis by CE-MS and investigating and improving interface methodologies using model problems. After this period of solving technical problems, CE-MS started to be used more and more for real-world applications in the protein field. An important aspect in this development is the increasing availability of high-resolution mass spectrometers, such as TOF and FTICR instruments, to researchers. This allowed performing protein mass detection with much higher precisions and accuracies. Until now, CZE has been the most frequently CE mode combined with MS. CZE separations can be set up rather straightforwardly using relatively simple BGEs and capillary coatings, and CZE-ESI-MS coupling meanwhile can be considered almost routine. CZE-ESI-MS has been used *e.g.* for protein identification/confirmation, the monitoring of protein changes like degradation or binding/unbinding of ligands, and glycoform analysis. An interesting field for CZE-ESI-MS, in which not much effort has been put yet, is the conformational analysis of proteins. Folding of proteins is particularly important for pharmaceutical proteins as it may affect its biological activity and/or immunogeneity. CZE-MALDI-MS is less frequently applied than CZE-ESI-MS, most likely due to the more complex interfacing and limited amount of

information which can be obtained from the mass spectrometric data. CZE-ICP-MS of metal-containing proteins has reached a stage of maturity now with several interfacing techniques available. ICP-MS provides an elegant means to reveal metal stochiometry in proteins, although obviously no other information on protein identity is obtained. CIEF is a very attractive technique for intact protein analysis, providing highly efficient protein separations and increased sensitivity through effective preconcentration. As is shown in this review, quite some efforts have been made to establish the coupling of CIEF and ESI-MS. Various researchers have shown the power of CIEF-ESI-MS, but the ampholytes causing background signals and ionization suppression of the proteins remain a hindrance for general application of the technique. As MALDI-MS is less susceptible towards the adverse effects of ampholytes, CIEF-MALDI-MS may provide a strong alternative for CIEF-MS. The use of ICP-MS detection for CIEF of metalloproteins might be an interesting option in order to avoid interferences from ampholytes, however, the development and use of this combination has not yet been reported so far. Recently, one can observe a clear trend towards the performance of protein separations on a chip format using microfluidics. Clearly, it would be advantageous to be able to use MS detection following these chip-based separations. First developments into this direction have been published and look promising. Still, more efforts and investigations will be needed to bring this technique to the level of more settled CE-MS methodologies. Overall, it can be concluded that the different CE-MS systems represent a powerful tool for the analysis of intact proteins yielding unique separations and information.

Table 2.1. CZE-ESI-MS of intact proteins.

Analytes[a]	Sample matrix[b]	BGE	Capillary coating[c]	Mass analyzer[d]	Remarks[e]	Ref.
Sheath-liquid interfacing						
Alb, CA, Cyt *c*, Hb, Myo, SOD	aqueous solution	1 M formic acid (pH 1.78)	no coating	QIT	optimization of tITP-CZE-ESI-MS parameters	[27]
apolipoproteins	aqueous solution	50 mM ammonium bicarbonate (pH 9.0), 15% acetonitrile	no coating	QTOF	investigation non-aqueous modifier in BGE	[54]
apomyoglobin	aqueous solution	1% formic acid	polybrene	magnetic sector	C_{18} preconcentration cartridge	[32]
aprotinin, CA, Cyt *c*, Lys, Myo, RNase A, α-CT, β-LGA, β-LGB	aqueous solution	20 mM ε-aminocaproic acid and 20 mM β-alanine (different pHs)	polyacrylamide	TQ	investigation sheath-liquid effects	[45]
aprotinin, CA, Cyt *c*, Myo	aqueous solution	10 mM acetic acid (pH 3.4)	aminopropylsilane	TQ	5-μm capillaries to obtain attomole LOD	[171]
aqueous humor proteins; urinary proteins	human aqueous humor; urine	1% formic acid	polybrene	magnetic sector	membrane and tITP preconcentration	[22]
aqueous humor proteins	human aqueous humor	1% acetic acid	polybrene	magnetic sector	membrane preconcentration	[31]
aqueous humor proteins	human aqueous humor	5% acetic acid, 2 mM ammonium acetate	polybrene	IT	membrane preconcentration	[34]
bacteriolysin, BSA, Cyt *c*, Hb, insulin, kallikrein	aqueous solution; human renal tissue	acetate (different compositions)	polyacrylamide	IT	optimization CZE-ESI-MS parameters	[43]
BSA, Myo, insulin, PLAP	aqueous solution	nonvolatile buffers of different compositions	no coating	IT	investigation nonvolatile buffers for CZE-ESI-MS	[53]
CA, Cyt *c*, Myo, RNase A, β-LGA	aqueous solution	20 mM ε-aminocaproic acid (pH 4.4)	polyacrylamide	TQ	comparison with CIEF-ESI-MS	[144]
CA, Cyt *c*, Myo, RNase, α-CT, α-LA	aqueous solution	different compositions	high-molecular mass charge-neutral polymer	SQ	new capillary coating	[172]
CA, insulin, rhHG, α-LA	pharmaceutical product	75 mM ammonium formate (pH 8.5)	polybrene-PVS bilayer	IT	characterization protein degradation products	[23]
Cyt *c*	aqueous solution	20 mM TRIS (pH 6.0)	permanently bonded polymer	IT	Separation and detection oxidized en reduced cytochrome *c*	[85]
Cyt *c*, HSA, Lys, Myo, RNase, α-CT	CSF	different compositions	Poly-LA 313	TOF	new capillary coating	[18]
Cyt *c*, HSA, Lys, β-LGA, α-CT, RNase A	CSF; plasma	10 mM acetic acid	PolyE-323	TOF	new capillary coating	[19]

Table 2.1. Continued

Cyt *c*, Lys, Myo, RNase A, TI, trypsin, α-CT, α-LA, β-LGB	aqueous solution; whey drink	different compositions	polystyrene nanoparticles	TQ	optimization buffer compositions for CZE-ESI-MS	[17]
Cyt *c*, Lys, Myo, RNase A, β-LGA, β-LGB	aqueous solution	10 mM ammonium acetate (pH 5.0)	polyacrylamide	TQ	ITP preconcentration	[30]
Cyt *c*, Lys, rhIL-6	aqueous solution	10 mM ammonium acetate (pH 4.2)	polyacrylamide	magnetic sector	ITP preconcentration; comparison with RPLC	[29]
Cyt *c*, Lys, RNase A	chicken egg; white wine; minced meat	75 mM ammonium acetate (pH 5.5)	EPyM-DMA	IT	new capillary coating	[21]
Cyt *c*, Myo	aqueous solution	20 mM TRIS (pH 8.25), 50 mM phosphate (pH 2.5), 10 mM ammonium acetate (pH 4.9)	no coating	TQ	ITP preconcentration	[173]
Cyt *c*, Myo, ubiquitin	aqueous solution	0.1% acetic acid	amine-coating	TOF	fast separations	[174]
GP 41, GR-LBP, Myo,	aqueous solution	0.5% formic acid	no coating	TQ	protein identification in the presence of polyethylene glycol	[175]
histones	aqueous solution	100 mM formic acid, 0.02% HPMC	HPMC	TQ	MALDI-TOF molecular mass conformation	[14]
human plasma antithrombin	aqueous solution	1 M acetic acid, 4 M urea	polyvinyl alcohol	QIT	glycoform characterization	[36]
IGF-I variants	aqueous solution	20 mM β-alanine (pH 3.8), 2,5% butanol, 3 mM DAPS	polyacrylamide	TQ	investigation non-aqueous modifier in BGE	[176]
mouse MT1	aqueous solution	30 mM acetate (pH 7.2)	no coating	TQ	monitoring zinc replacement for cadmium	[116]
Myo	aqueous solution	50 mM ammonium acetate (pH 8.3)	no coating	TOF	determination complex stability upon organic modifier addition	[44]
Myo, insulin	aqueous solution	TRIS (pH 8.4)	no coating	SQ	first report on CZE-ESI-MS of intact proteins	[84]
Myo, insulin	aqueous solution	10 mM TRIS (pH 8.3)	no coating	TQ	CE modification for CZE-ESI-MS	[177]
phycobiliproteins	*Spirulina platensis* microalgae	40 mM ammonium carbonate, acetonitrile, isopropanol (pH 7.8)	no coating	IT		[122]
phycobiliproteins	*Spirulina platensis* microalgae	40 mM ammonium carbonate, acetonitrile, isopropanol (pH 7.8)	no coating	IT	pressurized liquid extraction	[123]
polypeptides	snake venom	10 mM acetic acid (pH 3.5)	3-APS	magnetic sector		[178]
polypeptides, small proteins	human plasma	0.5% formic acid, 30% methanol	no coating	TOF	biomarker discovery hemodialysis patients with vitamin *c* supplementation; MALDI-TOF/TOF sequencing	[102]

Table 2.1. Continued

polypeptides, small proteins	human plasma	0.5% formic acid, 30% methanol	no coating	TOF	biomarker discovery ureamia	[103]
polypeptides, small proteins	human plasma; urine	0.5% formic acid, 30% methanol	no coating	TOF	polypeptide patterning in dialysates; comparison with other body fluids	[104]
polypeptides, small proteins	human urine	0.5% formic acid, 30% methanol	no coating	TOF, FTICR	polypeptide patterning; comparison different sequencing methods	[86]
polypeptides, small proteins	human urine	250 mM formic acid, 20% acetonitrile	no coating	TOF	biomarker discovery urethelial cancer; MALDI-TOF/TOF sequencing	[87]
polypeptides, small proteins	human urine	0.5% formic acid, 30% methanol	no coating	TOF	biomarker discovery neonatal UPJ obstruction; MALDI-TOF/TOF sequencing	[88]
polypeptides, small proteins	human urine	500 mM formic acid, 20% acetonitrile	no coating	TOF	biomarker discovery renal transplant rejection; MALDI-TOF/TOF sequencing	[90]
polypeptides, small proteins	human urine	250 mM formic acid, 20% acetonitrile	no coating	TOF	biomarker discovery prostate cancer; MALDI-TOF/TOF sequencing	[91]
polypeptides, small proteins	human urine	0.5% formic acid, 30% methanol	no coating	TOF	biomarker discovery diabetic nephropathy and albuminuria; MALDI-TOF/TOF sequencing.	[92]
polypeptides, small proteins	human urine	0.5% formic acid, 30% methanol	no coating	TOF	biomarker discovery diabetic nephropathy	[93]
polypeptides, small proteins	human urine	0.5% formic acid, 30% methanol	no coating	TOF	biomarker discovery IgA nephropathy; MALDI-TOF/TOF sequencing	[94]
polypeptides, small proteins	human urine	0.5% formic acid, 30% methanol	no coating	FTICR	biomarker discovery different renal diseases; FTICR-MS/MS sequencing	[95]
polypeptides, small proteins	human urine	0.5% formic acid, 30% methanol	no coating	TOF	biomarker discovery different renal diseases	[96]
polypeptides, small proteins	human urine	0.5% formic acid, 30% methanol	no coating	TOF	biomarker discovery MGN; comparison with SELDI	[97]
polypeptides, small proteins	human urine	0.5% formic acid, 30% methanol	no coating	TOF	biomarker discovery diabetic renal damage; MALDI-TOF/TOF sequencing	[98]
polypeptides, small proteins	human urine	0.5% formic acid, 30% methanol	no coating	TOF	biomarker discovery graft-versus-host disease; MALDI-TOF/TOF sequencing	[100]
polypeptides, small proteins	human urine	0.5% formic acid, 30% methanol	no coating	TOF	polypeptide patterning healthy persons; biomarker discovery MGN	[101]
polypeptides, small proteins	human urine; CSF	250 mM formic acid, 20% acetonitrile	no coating	TOF	biomarker discovery different diseases; MALDI-TOF/TOF sequencing	[89]

Table 2.1. Continued

polypeptides, small proteins	human urine; serum	0.5% formic acid, 30% methanol	no coating	TOF	biomarker discovery different diseases	[99]
rabbit liver MT isoforms	aqueous solution	100 mM acetic acid, 100 mM formic acid (pH 2.3)	no coating	TOF	MALDI for mass determination	[111]
rabbit MT, sheep MT, yeast MT	sheep liver extract; yeast cell filtrate	40 mM ammonium phosphate, isopropanol (pH 2.5)	no coating	IT		[115]
rat MT2	rat liver extract	100 mM formic acid, 2% methanol	no coating	TQ		[114]
rhEPO	pharmaceutical product	1 M acetic acid (pH 2.4), 20% methanol	polybrene	TOF	glycoform characterization	[106]
rhEPO	pharmaceutical product	1 M acetic acid (pH 2.4), 20% methanol and 2 M acetic acid	polybrene, polyacrylamide-based coating	TOF	glycoform characterization; glycan release and analysis	[107]
rhEPO	pharmaceutical product	1 M acetic acid (pH 2.4), 20% methanol	polybrene	TOF	glycoform characterization	[108]
rhEPO, bovine fetuin, AGP	aqueous solution	1 M acetic acid (pH 2.4), 20% methano and 2 M acetic acid	polybrene, polyacrylamide-based coating	TOF	glycoform characterization; glycan release and analysis	[105]
rhIL-6	aqueous solution	10 mM ammonium acetate (pH 4.2)	polyacrylamide	SQ	ITP preconcentration dimeric and monomeric forms; comparison with HPSEC	[28]
RNase B	aqueous solution	3 M formic acid	polybrene	TQ	glycoform characterization, comparison with other techniques	[179]
RNase B, POD	aqueous solution	2 M formic acid	polybrene, ethylene glycol	TQ	glycoform characterization	[110]
RNase B, rhBMP-2	aqueous solution	50 mM β-alanine (pH 3.5)	polyacrylamide	SQ	glycoform characterization	[109]
somatropins	aqueous solution	20 mM acetate (pH 9.0 and 10.0), acetonitrile	no coating	SQ		[180]
β_2-microglobulin	aqueous solution	100 mM ammonium acetate (pH 7.3)	no coating	TQ	LC-MS and CE-MS method development	[181]
Sheathless interfacing						
CA	aqueous solution	20 mM ammonium acetate (pH 8.3)	no coating	TQ	interface design: microdialysis membrane; post-separation acidification	[48]
CA, Cyt *c*, Myo, ubiquitin,	aqueous solution	10 mM acetate (pH 3.4, 7.9 and 9.0)	3-APS	TQ	new interface design: microdialysis junction	[47]
CA, Cyt *c*, ubiquitin	aqueous solution	10 mM acetic acid	3-APS	FT	sampling errors in small-bore capillaries	[182]

Table 2.1. Continued

CA, Cyt *c*, ubiquitin,	crude blood isolate	not specified	3-APS	FT	sub-attomole LOD	[183]
CA, Lys, TI	aqueous solution	10 mM ammonium acetate (pH 8.3 and 9.0)	polybrene	TQ	correlation charge in solution and in gas phase	[52]
cellular proteins	*E. coli* ribosomes	0.1% acetic acid, 50% acetonitrile	3-APS	IT	characterization proteins from *E. coli* ribosomes	[121
Cyt *c*, Hb, Lys, Myo, β-LGA	aqueous solution; whole blood	10 mM acetic acid (pH 3.4)	aminopropylsilane	TQ	interface design: in-capillary electrode;	[50]
Cyt *c*, Lys, Myo, α-LA	aqueous solution	0.1% acetic acid	3-APS	IT	new interface design: porous junction	[49]
Cyt *c*, Myo	aqueous solution	10 mM acetic acid (pH 3.2)	aminopropylsilane	TQ	interface design: in-capillary electrode	[51]
Cyt *c*, Myo, insulin	aqueous solution	10% and 5% acetic acid	no coating	TOF	off-line and on-line CZE-ESI-MS; gold-coated sheathless interface	[184]
Hb	red blood cells	0.1% acetic acid, 50% acetonitrile	3-APS	IT		[117]
Hb	red blood cells	10 mM acetic acid (pH 3.4)	aminopropylsilane	FTICR		[120]
Hb	single red blood cell	10 mM acetic acid (pH 3.4)	aminopropylsilane	TOF		[118]
Hb	single red blood cell	10 mM acetic acid (pH 3.4)	aminopropylsilane	FTICR		[119]
MT isoforms	rabbit liver extract	200 mM formic acid (pH 2.2)	polybrene / ethylene glycol	TQ	MT isoform characterization	[113]
polypeptides, small proteins	human serum	60 mM acetic acid, 20% methanol	poly-MAPTAC	TOF	biomarker discovery; tITP preconcentration	[185]

a. AGP: α1-acid glycoprotein, Alb: albumin, BSA: bovine serum albumin, CA: carbonic anhydrase, Cyt *c*: cytochrome *c*, Hb: hemoglobin, HSA: human serum albumin, IgA: immunoglobin A, IGF-I: human insulin-like growth factor I, Lys: lysozyme, MT: metallothionein, Myo: myoglobin, PLAP: placental alkaline phosphatase, POD: horse radish peroxidase, rhBMP-2: recombinant human bone morphogenetic protein-2, rhEPO: recombinant human erythropoietin, rhGH: recombinant human growth hormone, rhIL-6: recombinant human interleukin-6, RNase A: ribonuclease A, RNase B: ribonuclease B, SOD: superoxide dismutase, TI: trypsin inhibitor, α-CT: α-chymotrypsinogen A, α-LA: α-lactalbumin, β-LGA: β-lactoglobulin A, β-LGB: β-lactoglobulin B.

b. CSF: cerebrospinal fluid,*E. coli: Escherichia coli.*

c. 3-APS: 3-aminopropyltrimethoxysilane, EPyM-DMA: ethylpyrrolidine methacrylate-N,N-dimethylacrylamide, HPMC: hydroxypropylmethylcellulose,MAPTAC: (methacrylamido)propyl-trimethylammonium chloride, PVS: poly(vinylsulfonic acid).

d. FT: Fourier transform, IT: ion trap, TQ: triple quadrupole, QIT: quadrupole ion trap, QTOF: quadrupole time-of-flight.

e. HPSEC: high performance size exclusion chromatography, MGN: membranous glomerulonephritis, SELDI: surface-enhanced laser desorption ionization, tITP: transient isotachophoresis, UPJ: ureteropelvic junction.

Table 2.2. CZE-MALDI-TOF-MS of intact proteins.

Analytes[a]	Sample matrix	BGE	Capillary coating	Interface	MALDI matrix[b]	Remarks	Ref.
ApoAII, BSA, Cyt *c*, Lys, Myo,	aqueous solution	10 mM acetic acid (pH 3.4)	aminopropyl-trimethoxysilane	off-line sheath flow collector	CHCA	manual collection and spotting on MALDI target	[186]
BSA, LA, LG, Myo, RNase A, RNase B, substilin BPN	aqueous solution	10 mM CHES and 20 mM potassium chloride (pH 9.0)	no coating	off-line manual collection	sinapinic acid		[124]
CA, Cyt *c*, LF, Lys, RNase A, α-CT	aqueous solution; tear fluid	50 mM ammonium acetate (pH 7.4) and 10 mM acetic acid (pH 3.5)	PolyE-323	off-line sheath flow collector	sinapinic acid	direct mixing and deposition on MALDI target;	[125]
cachectic factor	human urine	20 or 60 mM sodium tetraborate	no coating	off-line manual collection	ferulic acid	glycoprotein identification	[127]
Cyt *c*, Lys	aqueous solution	25 mM acetate, phosphate and formate (pH 4.0)	DDAB	off-line fraction collection	CHCA	manual matrix mixing and spotting on MALDI target	[16]
Cyt *c*, Lys, Myo, Rnase A	aqueous solution	20 mM sodium acetate (pH 5.0)	polyarginine	off-line sheath flow collector	HCCA and 2,5-DHB	direct mixing and deposition on MALDI target	[62]
Cyt *c*, Lys, RNase A, β-LGA	aqueous solution	10 mM phosphate (pH 2.8)	polyethylene glycol	off-line membrane sample deposition	CHCA	continuous deposition on matrix-precoated membrane	[187]
DSPA α1, ovalbumin	aqueous solution	100 mM borate and 3 mM DAB (pH 8.5) 100 mM phosphate (pH 3.0)	bovine serum albumin for DSPA α1	off-line fraction collection	sinapinic acid	manual matrix mixing and spotting on MALDI target; glycoform characterization	[126]
insulin	aqueous solution	10 mM citric acid	polyvinyl alcohol	on-line continuous vacuum deposition	CHCA	matrix addition via liquid junction	[67]
insulin	aqueous solution	50 mM phosphate (pH 2.5)	no coating	on-line rotating ball	CHCA	matrix addition on ball, after sample deposition.	[188]
insulin, Myo, somatostatin	aqueous solution	30 mM sodium bicarbonate (pH 9.0)	no coating	off-line concentric flow deposition	2,5-DHB	direct mixing and deposition on MALDI target, FTICR detection	[63]
Lys	aqueous solution	20 mM ammonium acetate (pH 4.8)	no coating	off-line droplet containing MALDI matrix	sinapinic acid	sample concentration in matrix droplet, no separation	[189]
polypeptides, small proteins	human urine	30% methanol, 0.5% formic acid	no coating	off-line sheath flow collector	CHCA	direct mixing and deposition on MALDI target; comparison different sequencing methods; polypeptide patterning.	[86]

a. ApoAII: apolipoprotein AII, BSA: bovine serum albumin, BSA: bovine serum albumin, Cyt *c*: cytochrome *c*, DSPA α1: Desmodus plasminogen activator, LA: lactalbumin, LF: lactoferrin, LG: lactoglobulin, Myo: myoglobin, RNase A: ribonuclease A, RNase B: ribonuclease B, α-CT: α-chymotrypsinogen A, β-LGA: β-lactoglobulin A.

b. 2,5-DHB: 2,5-dihydroxybenzoic acid, CHCA: α-cyano-4-hydroxycinnamic acid.

Table 2.3. CZE-ICP-MS of intact proteins.

Analytes[a]	Sample matrix	Elements	BGE	Nebulizer[b]	Mass analyzer[c]	Remarks[d]	Ref.
A. hydrophila β-lactamase	aqueous solution	Zn, S	75 mM bis-Tris (pH 6.75)	MCN	sector field	stochiometry bound Zn	[190]
Alb, arginase, ConA, Tf	liver extract	Mn	10 mM TRIS (pH 8.3)	Meinhard	SQ	protein-Mn binding in liver	[137]
BSA, CA, CP, HTf, SOD	aqueous solution	Cd, Cu, Zn	25 mM borate (pH 9.2)	MCN	SQ	large volume sample stacking preconcentration	[25]
HSA	aqueous solution	Pt	15 mM phosphate (pH 7.4)	MCN	SQ	platinum-protein binding investigations	[138]
HSA, Tf	aqueous solution	Ru	15 mM phosphate (pH 7.4)	MCN	SQ	ruthenium-protein binding tumor-inhibiting agent	[139]
MT	fish liver cytosol	Cd, Cu, Zn	70 mM TRIS (pH 7.4), 5% methanol	HEN	SQ	large volume sample stacking preconcentration	[26]
MT	hepatopancreas cytosol	Cd	70 mM TRIS (pH 7.5), 5% methanol	HEN	SQ	full CZE-ICP-MS	[134]
MT	human brain cytosol	Cd, Cu, Zn	20 mM TRIS (pH 7.4)	MCN	sector field	SEC prefractionation; differentiation Alzheimer patients and healthy persons	[133]
MT	rat liver extract	Cd, Cu, Zn, S	20 mM TRIS (pH 7.4)	MCN	sector field	CZE-ESI-MS for unknown MT identification	[112]
MT	rat liver extract; rat kidney extract	Cd, Cu, Zn	12 mM TRIS (pH 7.5)	MCN	SQ	SEC prefractionation; comparison CZE/HPLC-ICP-MS and CZE/HPLC-ESI-MS	[132]
MT	*Synechococcus* extract	Cd	50 mM TRIS (pH 9.0)	MCN, CPN	SQ	SEC prefractionation	[136]
MT, MLP	bream liver cytosol	Cd, Cu, Zn, S	20 mM TRIS (pH 7.0-7.4)	MCN	Oc		[130]
MT, MLP	bream liver cytosol; Roe deer cytosol	Cd, Cu, Zn	100 mM Tricin (pH 7.2)	MCN	SQ		[129]
MT1, MT2, SOD	seal red blood cell extract	Cd, Cu, Zn	20 mM TRIS (pH 7.4)	MCN	Oc	comparison CE-ICP-MS and cLC-ICP-MS	[128]
rabbit liver MT, horse kidney MT	aqueous solution	Cd, Cu, Zn	50 mM TRIS (pH 9.0)	MCN, cross-flow	SQ	comparison different nebulizers for MT analysis	[77]
rabbit liver MT	aqueous solution	Cd, Cu, Zn	20 mM TRIS (pH 7.4)	MCN	sector field	AAMPS-coated capillary	[24]
rabbit liver MT	aqueous solution	Cd	70 mM TRIS (pH 7.4), 5% methanol	HEN, MCN	SQ	volatile species generation	[73]
rabbit liver MT	aqueous solution	Cd, Zn	70 mM TRIS (pH 7.4)	HEN, MCN	SQ, sector field	modification original nebulizer; comparison for MT analysis	[74]

Table 2.3. Continued

rabbit liver MT	aqueous solution	Cd, Zn	20 mM TRIS (pH 7.8)	MCN	SQ	modification original nebulizer	[78]
rabbit liver MT	aqueous solution	Cd, Cu, Zn	20 mM TRIS (pH 7.8)	ultrasonic	SQ	new CE-ICP-MS interface	[80]
rabbit liver MT	aqueous solution	Cd, Cu, Zn, S	20 mM TRIS (pH 7.4)	MCN	sector field		[131]
rabbit liver MT	aqueous solution	Cd, Cu, Zn	5 mM acetate (pH 6.0	MCN	SQ	CZE-ESI-MS for unknown MT identification	[135]
rabbit liver MT, horse spleen ferritin	aqueous solution	Cd, Cu, Zn, Fe	20 mM TRIS (pH 7.1),	concentric glass	SQ	new CE-ICP-MS interface	[71]
rabbit liver MT, horse spleen ferritin	aqueous solution	Cd, Cu, Zn, Fe	15 mM TRIS (pH 6.8)	MCN	SQ	frits in capillary; investigation suction effect interface	[76]
rabbit liver MT, horse spleen ferritin	aqueous solution	-	15 mM TRIS (pH 6.8)	OCN	SQ	frits in CE capillary for minimization interface suction;	[83]
rabbit MT	aqueous solution	Cd, Cu, Zn	50 mM TRIS (pH 9.1)	concentric tube, cross-flow	SQ	modification original nebulizer	[79]
rabbit MT	aqueous solution	Cd, Cu, Zn	50 mM TRIS (pH 9.1)	not clearly specified	SQ	potential error sources CZE-ICP-MS	[191]
rabbit MT; mouse MT	aqueous solution	Cd, Cu, Zn	12 mM TRIS (pH 7.5)	MCN	SQ	modification original nebulizer; metal-binding experiments	[75]
Tf isoforms	aqueous solution	Fe	15 mM borate (pH 8.5), 3 mM DAB	MCN	Oc	comparison CE-ICP-MS and HPLC-ICP-MS	[192]

a. A. hydrophila: Aeromonas hydrophila, Alb: albumin, BSA: bovine serum albumin, CA: carbonic anhydrase, ConA: concanavalin A, CP: ceruloplasmin, HSA: human serum albumin, HTf: holotransferrin, MLP: metallothionein-like proteins, MT: metallothioneins, SOD: superoxide dismutase, Tf: transferrin.

b. CPN: concentric pneumatic nebulizer, HEN: high efficiency micronebulizer, MCN: microconcentric nebulizer, OCN: oscillating capillary nebulizer.

c. Oc: octopole, SQ: single quadrupole.

d. AAMPS: 2-acrylamido-2-methyl-1-propanesulfonic acid, cLC: capillary liquid chromatography.

Table 2.4. CIEF-ESI-MS of intact proteins.[a]

Analytes[b]	Sample matrix[c]	Ampholytes	Capillary coating	Mass analyzer[d]	Remarks[e]	Ref.
BSA, CA, Cyt *c*, Myo, insulin, RNAse A, β-LG	aqueous solution	1,25% eCap CIEF (p*I* 3-10)	neutral (not specified)	QTOF	CIEF-RPLC-MS for ampholyte removal and additional separation	[61]
CA isozymes	aqueous solution	1% Pharmalyte (p*I* 5-8)	polyvinyl alcohol	magnetic sector	microdialysis junction for ampholyte removal	[58]
CA, Cyt *c*, Myo	aqueous solution	0.5% Pharmalyte (p*I* 3-10)	polyacrylamide	TQ	first CIEF-ESI-MS publication	[57]
CA, Cyt *c*, Myo	aqueous solution	0.5% Pharmalyte (p*I* 3-10)	polyacrylamide	TQ	microdialysis acidification	[193]
CA, Cyt *c*, Myo, RNase A, β-LGA	aqueous solution	0.5% Pharmalyte (p*I* 3-10)	polyacrylamide	TQ	comparison with CZE-ESI-MS	[144]
CA, Lys, Myo, TI, CSF proteins	aqueous solution, human CSF	1% Servalyte (p*I* 3-10) and Isolyte (p*I* 3-10) mixture	polyvinyl alcohol	magnetic sector	on-line desalting and protein identification	[194]
CA, Myo, β-LGA	aqueous solution	1% Pharmalyte (p*I* 5-8)	polyvinyl alcohol	SQ	microdialysis junction for ampholyte removal	[59]
CA, Myo, β-LGB	aqueous solution	5% Pharmalyte (p*I* 5-8)	siloxanediol polyacrylamide	SQ	CIEF-FFE chip-ESI-MS for ampholyte removal	[195]
CA, Tf, β-LGB	aqueous solution	1% Pharmalyte (p*I* 3-10)	polyacrylamide	TOF	quantitative analysis of proteins	[55]
cellular proteins	Soluble fraction from *S. cerevisiae* cell lysate	1% Pharmalyte (p*I* 3-10), 4 M urea and 2 M thiourea	hydroxypropyl cellulose	QTOF	CIEF-RPLC-ESI-MS for ampholyte removal and additional separation	[15]
cellular proteins	*E. coli* cell lysate; *D. radiodurans* cell lysate	0.5% Pharmalyte (p*I* 3-10)	polyacrylamide	FTICR	overview own work	[20]
cellular proteins	*E. coli* cell lysate	0.5% Pharmalyte (p*I* 3-10) and Pharmalyte (p*I* 5-8) mixture	polyacrylamide	TQ	2-D mapping cell lysate	[56]
cellular proteins	yeast enzyme concentrate type II	1% eCap CIEF (p*I* 3-10), 6 M urea and 100 mM DTT	neutral (not specified)	QTOF	CIEF-RPLC-ESI-MS for ampholyte removal and additional separation	[60]
cellular proteins	*E. coli* cell lysate; *S. cerevisiae* cell lysate	0.5% Pharmalyte (p*I* 3-10)	polyacrylamide	FTICR	identification due to incorporation isotope labeled amino acids	[150]
cellular proteins	E. coli cell lysate	0.5% Pharmalyte (p*I* 3-10)	polyacrylamide	FTICR, QIT	stepwise mobilization for increased resolution	[151]
cellular proteins	E. coli cell lysate	0.5% Pharmalyte (p*I* 3-10)	polyacrylamide	FTICR	identification due to incorporation isotope labeled amino acids	[152]

Table 2.4. Continued

cellular proteins	*E. coli* cell lysate; *D. radiodurans* cell lysate	0.5% Pharmalyte (p*I* 3-10)	polyacrylamide	FTICR	growth in isotope labeled media	[153]
cellular proteins	*E. coli* cell lysate	0.5% Pharmalyte (p*I* 3-10)	polyacrylamide	FTICR	variation protein expression upon stress; growth in isotope depleted media	[154]
cellular proteins	*E. coli* cell lysate	0.5% Pharmalyte (p*I* 3-10)	polyacrylamide	FTICR	growth in isotope depleted media	[155]
CPK, GAPDH, Hb, lentil lectin, β-LGA	aqueous solution	1% Pharmalyte (p*I* 3-10)	polyacrylamide	FTICR	separation and identification noncovalent protein complexes	[142]
Cyt *c*, Hb variants A and S, Myo, β-LGA,	aqueous solution	1% Pharmalyte (p*I* 3-10) and Pharmalyte (p*I* 5-8)	polyacrylamide	QIT	isoform analysis	[196]
Hb variants A, C, S and F	aqueous solution	0.5% Pharmalyte (p*I* 5-8)	polyacrylamide	TQ		[141]
Hb variants A, C, S and F	aqueous solution	1% Pharmalyte (p*I* 3-10), Ampholine (p*I* 3.5-10) and Servalyte (p*I* 3-10) mixture	polyvinyl alcohol	TQ	active capillary positioning interface	[147]
Hb variants A, C, S and F	*E. coli* cell lysate	0.5% Pharmalyte (p*I* 3-10) and Pharmalyte (p*I* 5-8) mixture	polyacrylamide	FTICR	first CIEF-ESI-FTICR publication	[149]
Hb variants A, C, S and F, CSF proteins	human whole blood; CSF	1% Pharmalyte (p*I* 3-10) and Isolyte (p*I* 3-10) mixture	polyvinyl alcohol	magnetic sector	on-line desalting and protein identification	[145]
Hb variants A, C, S and F, Myo, β-LG	aqueous solution	0.5% Pharmalyte (p*I* 3-10) and 0.5% Pharmalyte (p*I* 5-8)	polyacrylamide	TOF		[146]
human ADH isoenzymes	aqueous solution	1% Pharmalyte (p*I* 3-10)	polyacrylamide	FTICR	separation and identification noncovalent protein complexes	[197]
RNase A	aqueous solution	0.5% Pharmalyte (p*I* 3-10) and 0.05% TEMED	polyacrylamide	TQ	monitoring protein refolding	[143]
Src homology 2	aqueous solution	1% Ampholine (p*I* 3.5-10)	polyvinyl alcohol	QIT	determination peptide binding to protein	[198]
Tf	aqueous solution	0.5% Pharmalyte (p*I* 5-8)	polyacrylamide	TQ	glycoform characterization	[199]

a. sheath-liquid interfaces were used.

b. ADH: alcohol dehydrogenase, BSA: bovine serum albuumin, CA: carbonic anhydrase, CA: carbonic anhydrase, CPK: creatine phosphokinase, CSF: cerebrospinal fluid, Cyt *c*: cytochrome *c*, GAPDH: glyceraldehyde-3-phosphate dehydrogenase, Hb: hemoglobin, Lys: lysozyme, Myo: myoglobin, RNase A: ribonuclease A, Tf: transferrin, TI: trypsin inhibitor, β-LG: β-lactoglobulin, β-LGA: β-lactoglobulin A, β-LGB: β-lactoglobulin B.

c. *D. radiodurans: Deinococcus radiodurans, E. coli: Escherichia coli, S. Cerevisiae: Saccharomyces cerevisiae*.

d. SQ: single quadrupole, TQ: triple quadrupole , QIT : quadrupole iontrap, QTOF: quadrupole time-of-flight.

e. FFE: free-flow electrophoresis.

Table 2.5. CIEF-MALDI-TOF-MS of intact proteins.[a]

Analytes[b]	Sample matrix	Ampholytes	Capillary coating	MALDI matrix	Remarks	Ref.
CA, Hb variants, Myo, β-LGA	aqueous solution	1% Pharmalyte, Ampholine and Servalyte mixture(p*I* 3-10)	applied, but not specified	sinapinic acid	manual spotting	[140]
CA, Lys, Myo	aqueous solution	5% Pharmalyte (p*I* 5-8) and Ampholytes (p*I* 5-7) mixture	siloxanediol-polyacrylamide	sinapinic acid	direct deposition on MALDI target	[157]
CA, Myo, Oval, β-LGA,	aqueous solution	2% Ampholine (p*I* 3.5-10)	polyvinyl alcohol	sinapinic acid	manual spotting; wide-bore capillaries	[156]
blood serum proteins	human blood serum	2.5% Pharmalyte (p*I* 3-10)	applied, but not specified	sinapinic acid	direct deposition on MALDI target; 2-D mapping of serum proteins	[158]

a. off-line sheath-liquid collectors were used.

b. CA: carbonic anhydrase, Hb: hemoglobin, Lys: lysozyme, Myo: myoglobin, Oval: ovalbumin, β-LGA: β-lactoglobin A, β-LGA: β-lactoglobulin.

Table 2.6. Chip-based electrophoresis-MS of intact proteins.

CE mode	Analytes[a]	Sample matrix	BGE/ampholytes	Interface	Mass analyzer[b]	Remarks	Ref.
CZE	Cyt *c*, Myo, β-LGA, β-LGB	aqueous solution	20 mM ε-aminocaproic acid (pH 4.4)	liquid junction ESI	QIT		[162]
CZE	hTRF2 DBD, Myo, Lys	aqueous solution	50 mM ammonium acetate (pH 5.7)	liquid junction ESI	QTOF	top-down analysis of proteins; PolyE-323-coated capillary	[163]
CIEF	BSA, Lys, Myo, RNase A, TI	aqueous solution	1% Pharmalyte (p*I* 3-10)	on-chip MALDI (matrix: sinapinic acid)	TOF	chip material optimization	[166]
CIEF	CA, Myo	aqueous solution	1% Pharmalyte (p*I* 3-10)	sheath-liquid ESI	QIT		[164]
CIEF	CA, Myo, TI	aqueous solution	1% Pharmalyte (p*I* 3-10)	on-chip MALDI (matrix: not specified)	TOF		[165]
CIEF	CP, TI	aqueous solution	2% CIEF ampholytes (p*I* 3-10)	on-chip MALDI (matrix: sinapinic acid)	TOF	freeze drying fixation	[161]

a. CA: carbonic anhydrase, CP: creatine phosphokinase, Cyt *c*: cytochrome *c*, hTRF2 DBD: human TTAGGG repeat binding factor 2 DNA-binding domain, Lys: lysozyme, Myo: myoglobin, RNase A: ribonuclease A, TI: trypsin inhibitor, β-LGA: β-lactoglobin A, β-LGB: β-lactoglobin B.

b. QIT: quadrupole iontrap, QTOF: quadrupole time-of-flight.

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New developments and applications in the period 2007-2010

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Abstract

Capillary electrophoresis (CE) coupled to mass spectrometry (MS) has proven to be a powerful analytical tool for the characterization of intact proteins, as it combines the high separation efficiency of CE with the selectivity of MS. This review provides an overview of the development and application of CE-MS methods within the field of intact protein analysis as published between January 2007 and June 2010. Ongoing technological developments with respect to CE-MS interfacing, capillary coatings for CE-MS, coupling of capillary isoelectric focusing (CIEF) with MS, and chip-based CE-MS are treated. Furthermore, CE-MS of intact proteins involving electrospray ionization (ESI), matrix assisted laser-desorption ionization (MALDI) and inductively coupled plasma ionization (ICP) is outlined, and overviews of the use of the various CE-MS methods are provided by tables. Representative examples illustrate the applicability of CE-MS for the characterization of proteins, including glycoproteins, biopharmaceuticals, protein-ligand complexes, biomarkers and dietary proteins. It is concluded that CE-MS is a valuable technique with high potential for intact protein analysis, providing useful information on protein identity and purity, including modifications and degradation products.

1. Introduction

Advancements in the fields of protein chemistry, biotechnology and biopharmaceuticals have increased the demand for techniques that are suitable for the analysis of intact proteins. In various instances, like *e.g.* during quality control, it can be important to be able to characterize a protein without affecting its primary structure and conserving potentially present modifications. Establishment of the protein integrity as actually present in a sample can be essential and possible artifacts as result of derivatization or protein digestion should be avoided. In order to analyze complex protein mixtures or to distinguish between highly related protein species, an efficient separation is a prerequisite. Also to increase the reliability of (selective) detection, a preceding separation can be critical.

Next to more conventional analytical techniques, like liquid chromatography, size-exclusion chromatography and gel electrophoresis [1], capillary electrophoresis (CE) has proven to be a powerful separation technique for intact proteins [2]. The separation efficiency of CE can be extremely high for large molecules, such as proteins, as longitudinal diffusion – ideally the predominant contributor to peak broadening in CE – is relatively low. CE analyses can be carried out under mild conditions without the need of organic solvents or very high salt concentrations. This enables the study of proteins without causing conformational changes or protein degradation during analysis. Many posttranslational modifications like glycosylation or phosphorylation can potentially change the electrophoretic mobility of a protein, and can therefore be differentiated with CE. Mass spectrometry (MS) has developed into one of the most popular and useful detection techniques in separation sciences [3]. MS is well suited for protein analysis because of its sensitivity and selectivity, and due to its ability to characterize proteins. MS can considerably enhance the utility of CE by providing information about the identity of the separated compounds. Therefore, coupling of CE to MS creates a powerful analytical tool for the characterization of intact proteins.

Coupling of CE with MS is less straightforward than combining liquid chromatography and MS. Nevertheless, since the introduction of CE-MS in the late 80's of the past century [4,5], MS has gained importance as a selective detection technique for CE. Over the years, various interfaces for several types of ionization sources have been developed. For CE-MS of proteins, electrospray ionization (ESI), matrix assisted laser-desorption ionization (MALDI) and inductively coupled plasma ionization (ICP) have been used. ESI is the predominant ionization technique applied in CE-MS of proteins. Generally, CE-MS interfacing via an ESI source can be performed in two different ways. In the most frequently applied approach, the interface uses a sheath liquid that mixes with the CE effluent as it exits the separation capillary. The sheath flow serves to establish the electrical contact with the background electrolyte (BGE) and facilitates ESI applying a nebulization gas. In the second, sheathless CE-ESI-MS approach, the terminating electrical contact for the CE process is made directly to the BGE just before or after it leaves the capillary using, for example, a conductive coating or a microelectrode inserted in the CE capillary. ESI produces multiply charged ions

in the gas phase. As a result, a so-called protein charge envelope comprising ions with mass-over-charge ratios of typically 500-3000 will be obtained. Protein molecular masses can be determined by deconvolution of the ESI mass spectrum. When employing mass spectrometers with sufficient mass resolution and accuracy, protein modifications, like *e.g.* glycosylation [6] and oxidation [7], can be determined. Furthermore, information on the conformation of proteins (native vs. denatured) can be also obtained by studying the shape and position of the charge envelope [8]. In CE-ESI-MS, the applied BGEs preferably should be volatile (*e.g.* formic acid or ammonium acetate) and of low concentration to avoid protein ionization suppression and background signals.

Combination of CE with MALDI-MS and ICP-MS requires specific interfaces. MALDI is very well suited for the analysis intact proteins. However, as MALDI usually should be carried out in vacuum, it is quite difficult to perform CE-MALDI-MS in an on-line fashion. Furthermore, for effective ionization the proteins have to be co-crystallized on a surface with a UV absorbing matrix on a surface prior to analysis. Therefore, most CE-MALDI-MS systems involve stepwise deposition of the CE effluent on a MALDI plate. After protein fractions are collected on the plate, it is transferred into the MALDI source and the deposited compounds are desorbed and ionized by the laser. MALDI generally results in the formation of singly or doubly charged protein molecules allowing determination of molecular mass. Adduct formation may complicate actual protein mass determinations. Interestingly, MALDI is more tolerant to sample constituents like buffers and salts than ESI, and, therefore, non-volatile BGEs can be used for CE-MALDI-MS. On the other hand, MALDI-MS is sensitive to the quality of the crystallization of the matrix and the compounds. The analyte is often not uniformly distributed among or within the matrix crystals. Furthermore, the choice of matrix and matrix additives has been shown to significantly influence the MALDI-MS response of proteins [9].

ICP ionization takes place in torch operating at 5000-9000 K. To achieve CE-ICP-MS, the CE effluent is sprayed into a heated chamber with the aid of a nebulization gas. CE-ICP-MS interfacing often involves the use a sheath liquid in order to allow completion of the electrical circuit for CE and to stabilize the formation of the aerosol. During ICP ionization proteins are completely broken down into their charged elements, typically in the M^+ state, which are then detected by MS. CE-ICP-MS is commonly used for the selective analysis of proteins containing metal ions or other heavy elements. Due to the destructive detection principle, no information on the molecular mass of a protein can be obtained with ICP-MS detection. As a consequence, separation of proteins prior to ICP-MS detection is essential in order to distinguish different proteins in one sample.

A considerable number of papers reporting on the development and application of CE-MS for intact protein analysis using ESI, MALDI or ICP ionization have appeared since our previous review on this topic [10]. The present update provides an overview of the advancements made between January 2007 and June 2010, giving discussions, tables and

illustrative examples. The paper is confined to CE-MS studies in which intact protein molecules are separated, that is, in which proteins are not deliberately broken down in peptides or derivatized prior to analysis. Protein modifications as result of, *e.g.*, degradation or glycosylation are covered by the review. As there are excellent reviews covering CE-MS interfacing using various ionization techniques [11-14] and capillary coatings [15-17], this paper will address these topics only briefly focusing on recent technological developments for protein analysis.

2. CE-ESI-MS

2.1 Technological developments

2.1.1 Interfacing

CE-ESI-MS can be performed with either a sheath-liquid or a sheathless interface. With the sheathless interface the initial droplets formed during the electrospray process are small leading to more efficient ionization when compared to sheath-liquid interfacing [18]. Optimized sheathless interfaces offer the highest sensitivities in CE-MS, but until now they have not been frequently used for CE-MS analysis of proteins, most likely due to their limited robustness, and lack of commercial availability. Recently, some interesting new designs of CE-ESI-MS interfaces have been described that provide improved stability [19,20]. Maxwell *et al*. introduced an interface that uses a tapered and beveled stainless steel hollow needle surrounding the separation capillary terminus (Figure 2.14A) [19]. In this way, the inside of the needle serves as the CE outlet vial and the outside tip acts as the electrospray emitter. A chemical modifier solution is introduced through a second capillary connected to the needle via a tee junction. The presence of the modifier solution allowed the use of neutral-coated capillaries for protein analysis by CE-MS without the application of an assisting pressure, normally needed in the absence of electroosmotic flow (EOF). Three model proteins were separated within 14 min using a neutral coated capillary and an acidic BGE, and good quality mass spectra for were obtained for the proteins. Some protein peak tailing was observed, but this was attributed to protein interaction with the capillary wall and not to the interface.

Moini introduced a porous tip sheathless CE-MS interface [20]. In this design, the last 3-4 cm of the fused-silica capillary are etched with hydrofluoric acid producing a 5-µm thick porous wall that is conductive when in contact with an electrolyte (Figure 2.14B). The porous capillary outlet protrudes from a stainless steel ESI needle filled with static conductive liquid allowing electrical contact and electrospray formation at the capillary tip. Haselberg *et al*., showed that with this sheathless interface, detection limits for four model proteins were improved by a factor 50 to 140 compared to sheath-liquid CE-MS, leading to sub-nM detection limits [21]. The potential usefulness of the porous tip sprayer for protein analysis was demonstrated by the analysis of protein-protein and protein-metal complexes [22].

Carbonic anhydrase and hemoglobin from red blood cells were separated using a BGE of ammonium acetate (pH 7.4). Due to the absence of sheath liquid, which normally contains considerable amounts of organic solvent, CE-MS could be performed under native conditions, thereby keeping the carbonic anhydrase-zinc protein-metal complex and the tetrameric hemoglobin protein-protein complex intact.

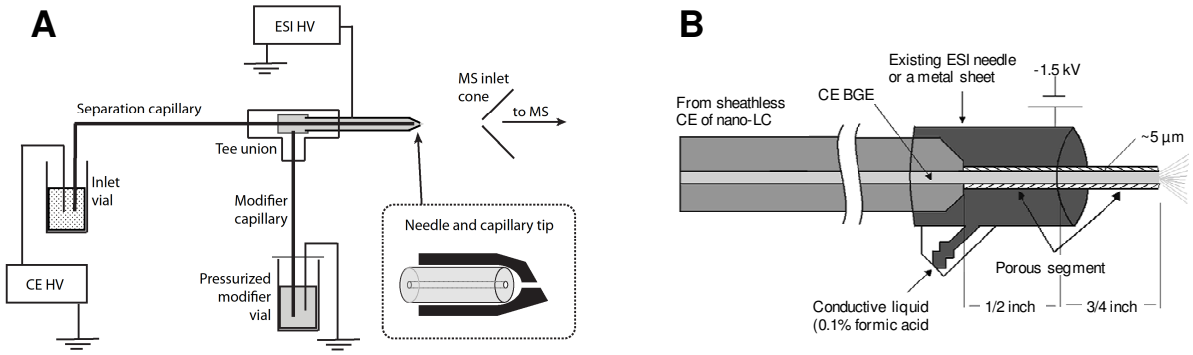

Figure 2.14. Schematic illustration of (A) the interface introduced by Maxwell *et al* [19], including a dissected view of needle tip with inserted capillary in the inset and (B) the interface with a porous tip capillary positioned in a stainless steel capillary as described by Moini.

2.1.2 Capillary coatings

A typical problem in CZE of proteins, and especially basic proteins, is that the separation is often hampered by adsorption of the proteins onto the fused-silica capillary wall due to electrostatic and hydrophobic interactions. In order to allow efficient CZE separations of proteins, coating of the inner capillary surface is often needed to minimize or completely prevent protein-wall interactions. In order to achieve efficient ESI-MS detection, coatings should be permanently attached to the capillary wall to avoid suppression of analyte ionization, background signals, and/or contamination of the ion source and MS optics by coating agents. Furthermore, an appreciable and constant EOF is preferred to achieve adequate and reproducible interfacing conditions.

As is evident from Section 2.2 and Table 2.7, a large number of recently reported CE-ESI-MS studies utilize capillary coatings to achieve proper protein separation and detection. Various capillary coating approaches have also been newly introduced to CE-MS of proteins over the past years [23-26]. Elhamilli *et al.* described the use of a monoquaternarized piperazine – which was first introduced by Rigetti *et al.* [27] – as a coating agent for CE-MS [23]. The coating agent reacts at alkaline pH with the silanol groups present on the capillary wall to form a covalent coating that showed no bleeding of the coating agent in the mass spectrometer. The charge of the capillary surface is reversed and a high and stable anodic EOF is obtained. This coating was used for the CE-MS analysis of four basic proteins. The proteins were separated within 6 min with efficiencies up to 125,000 theoretical plates. Haselberg *et al.* demonstrated the usefulness of a positively-charged noncovalent triple-layer

coating, which was developed by Katayama *et al*. [28], for the analysis of basic proteins by CE-MS [24]. The coating was prepared by successively flushing the capillary with solutions of Polybrene, dextran sulfate and Polybrene. The feasibility of using the coated capillaries for CE-MS was shown by the analysis of a llama antibody sample. Garza *et al*. used a BGE containing either Polybrene or polyE-323 to dynamically coat the CE capillary [25]. As the coating agents are present in the BGE, the coating is continuously regenerated and stable migration times were obtained. Six model proteins were baseline separated within 18 min with both dynamic coatings without any noticeable ionization suppression. The Polybrene dynamic coating also proved suitable for the untargeted CE-MS analysis of a mixture containing 55 ribosomal proteins, obtaining a partial separation. For one selected protein it was demonstrated that a narrow peak was obtained in this CE-MS system using a dynamic coating. Fermas *et al*. demonstrated the compatibility of a neutral polyethylene oxide coating for the CE-MS analysis of basic proteins [26]. Three model proteins were separately analyzed and good quality mass spectra were obtained. However, an additional pressure applied to the CE capillary was required during CE-MS analysis to obtain a stable electrospray.

2.1.3 Capillary isoelectric focusing

CIEF-ESI-MS has been mainly used for proteome analysis and the characterization of hemoglobin variants. However, the ampholytes used to separate proteins in CIEF are not volatile. Upon mobilization, these ampholytes may enter the MS ion source. As a result, often background signals and ionization suppression of the proteins is observed, hindering general application of the technique. Mokkadem *et al*. developed an improved protocol for the on-line coupling of CIEF with ESI-MS [29]. The method comprises a discontinuous filling of the capillary with a volatile catholyte (60% of capillary volume) and a sample-ampholyte mixture (40% of capillary volume). The ampholyte concentration was optimized in order to minimize ionization suppression. To obtain sufficient separation and still good signal-to-noise ratios for six model proteins, an ampholyte concentration of 1.5% was required (Figure 2.15A-C). Using glycerol in the sample/ampholyte mixture kept hydrophobic proteins solubilized and allowed the use of unmodified bare fused-silica capillaries, as the glycerol/water medium strongly reduced EOF. Glycerol was found to be compatible with MS detection and good quality mass spectra were obtained with only minor background signals of the ampholytes (Figure 2.15D).

An approach to prevent ampholytes to reach the MS ion source, as previously reported by Zhou *et al*., is the coupling of CIEF to reversed-phase liquid chromatography (RPLC) ESI-MS for the analysis of intact proteins [30]. After focusing of proteins with CIEF, different p*I* fractions are transferred to storage loops. Subsequently, the fractions are led one by one to a RPLC system, where comigrating proteins are separated from each other and, more importantly, from the ampholytes that might cause ionization suppression. This system was used for the analysis of a *Chlorobium tepidum* lysate [31]. About 1200 individual proteins and

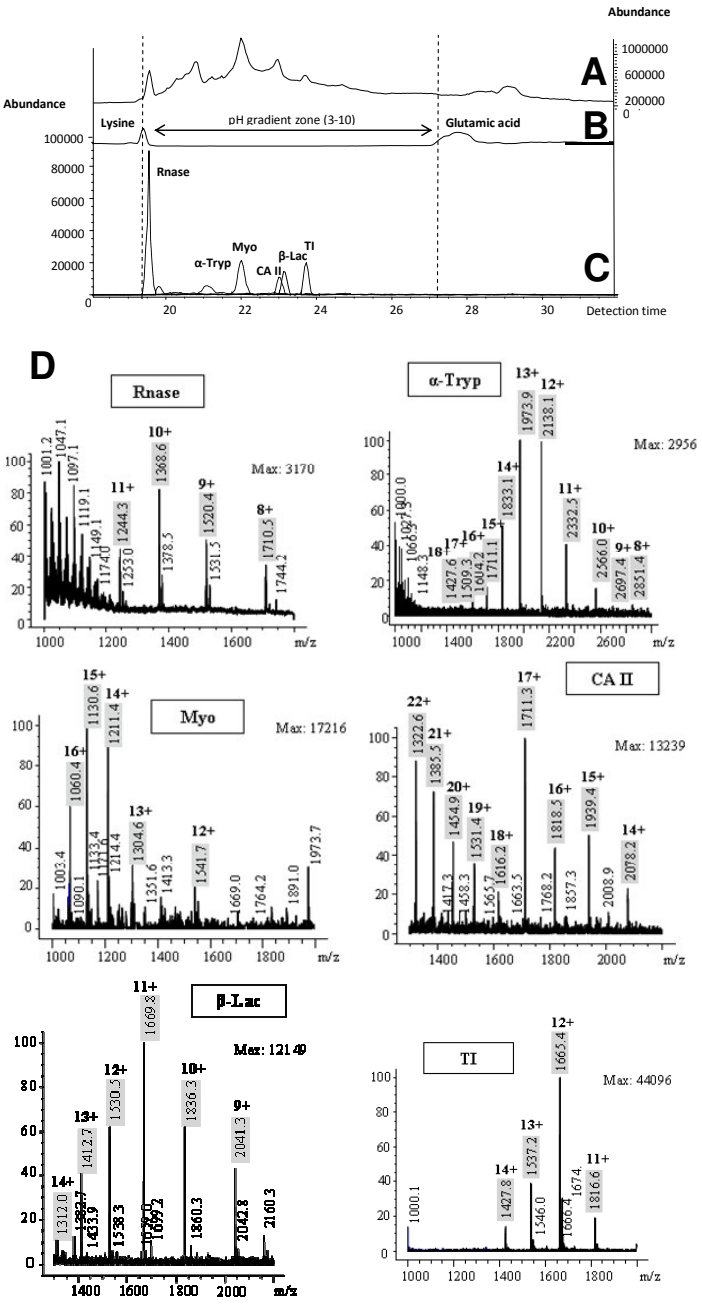

Figure 2.15. (A) TIE of the CIEF-ESI-MS analysis of six intact proteins. (B) EIE at *m/z* 148 to visualize lysine and glutamic acid, which indicate the beginning and end of the pH gradient. (C) EIEs of ribonuclease (Rnase), α-trypsinogen (α-Tryp), myoglobin (Myo), carbonic anhydrase II (CA II), β-lactalbumin (β-Lac) and trypsin inhibitor (TI). (D) Mass spectra of the six proteins as obtained through the peaks [29].

polypeptides were detected in a sequence that lasted less than 8 h. The p*I* vs. M_w profile obtained from CIEF-RPLC-MS compared favorably with theoretical data derived from the *Chlorobium tepidum* genome and experimental data obtained from 2D-PAGE.

2.1.4 Chip-based electrophoresis

Recently, there has been a growing interest in the development of chip-based analytical systems, as they may increase analysis speed and performance. Sikanen *et al.*

introduced a fully microfabricated and monolithically integrated CZE-ESI chip for coupling with MS [32]. The chips were fabricated by a standard lithographic process. As the coaxial sheath flow interface was patterned as an integral part of the chip, the fluidic design was dead-volume free. No significant peak broadening occurred so that very narrow peak widths were obtained. The applicability for intact protein analysis was demonstrated by the analysis of myoglobin. To avoid unwanted interactions between the wall of the chip and the protein, 1-methoxy-2-propyl acetate was added to the BGE as a dynamic coating.

Mellors *et al*. developed a similar microfabricated device in which ESI was performed directly from the corner of a rectangular glass microchip [33] (Figure 2.16A and 2.16B). An electrokinetic-based hydraulic pump was integrated on the chip that directed separated compounds to the monolithically integrated spray tip. A positively charged surface coating

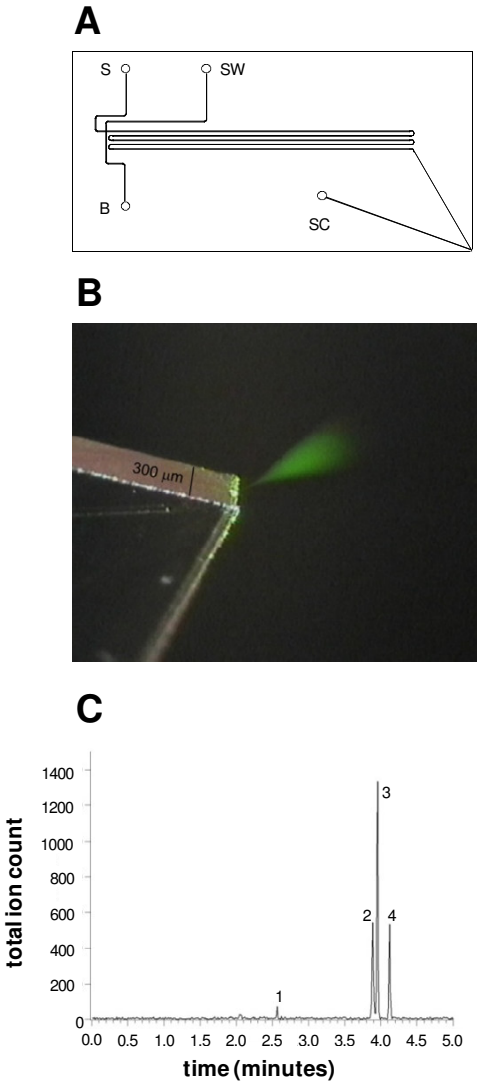

Figure 2.16. (A) Schematic representation of a CE-ESI-MS chip design for intact protein analysis. The length of the separation channel was 20.5 cm from the injection cross to the outlet. The reservoirs are labeled S (sample), B (buffer), SW (sample waste) and SC (side channel). (B) Image of the electrospray plume generated from the corner of the CE-ESI-MS chip. (C) TIE for the chip CE-ESI-MS analysis of three standard proteins. The peaks are (1) heme group of myoglobin, (2) ribonuclease A, (3) myoglobin, and (4) cytochrome *c* [33].

(PolyE-323) was used to prevent protein-surface interactions. The device was used to perform CE-MS analysis of three intact model proteins in less than 5 min with efficiencies over 280,000 theoretical plates (Figure 2.16C). In a following publication the usefulness of this chip CE-MS set-up for the analysis of hemoglobin from single cells was demonstrated [34]. Individual erythrocytes were lysed at the beginning of the separation channel. Subsequently, the content of the cell was separated with chip CE and detected with ESI-MS. The BGE caused hemoglobin to dissociate into its α- and β-subunits. As a result, the noncovalently bound heme was released from the protein structure. By monitoring the mass trace of the heme group (*m/z* 616), the presence of hemoglobin released from the individual cells was detected.

2.2 Applications

An overview of recent CE-ESI-MS applications is provided in Table 2.7 (p. 86). In the following paragraphs some typical examples are outlined, focusing on glycoproteins, biopharmaceuticals, protein-ligand complexes, biomarkers and dietary proteins.

2.2.1 Glycoproteins

Glycosylation is an important parameter in defining biological and biophysical properties of proteins. Oligosaccharides are attached to the proteins posttranslationally and therewith glycosylation is a major source of protein heterogeneity. The carbohydrate structure plays an important role in determining biological activity. The first protein with a complex glycosylation pattern which has been extensively analyzed with CZE-ESI-MS, is recombinant human erythropoietin [35]. Currently, CZE-ESI-MS has found application for the analysis of glycosylation of various proteins [6,36-39]. Neutral [36,37], negatively-charged [38], and positively-charged [6,39] coatings were used to (partially) separate the different protein isoforms. With the negatively-charged coating a high-pH BGE was required to prevent protein adsorption to the wall, while the other coatings were used in combination with a low-pH BGE. A typical example of glycoprotein analysis by CZE in combination with high-resolution TOF-MS, as described by Puerta and Bergquist, is the analysis of vascular endothelial growth factor 165 ($VEGF_{165}$) [6]. VEGF165 is a putative biomarker and is considered as a promising therapeutic drug. An in-house developed polycationic polymer coating (Poly-LA 313), which has been characterized previously using model proteins, in combination with a low-pH formic acid BGE was found suitable for the separation of isoforms of recombinant human $VEGF_{165}$ expressed in Spodoptera frugiperda (Figure 2.17A). At least seven partially separated protein species were detected. However, mass spectra showed complexity as different series of protein charge envelopes were obtained within a single electrophoretic peak (Figure 2.17B). After deconvolution of the mass spectra 16 glycoforms of intact $VEGF_{165}$ were identified (Figure 2.17C). The glycosylation of this protein is mainly based on biantennary structures consisting of hexose (Hex) and N-

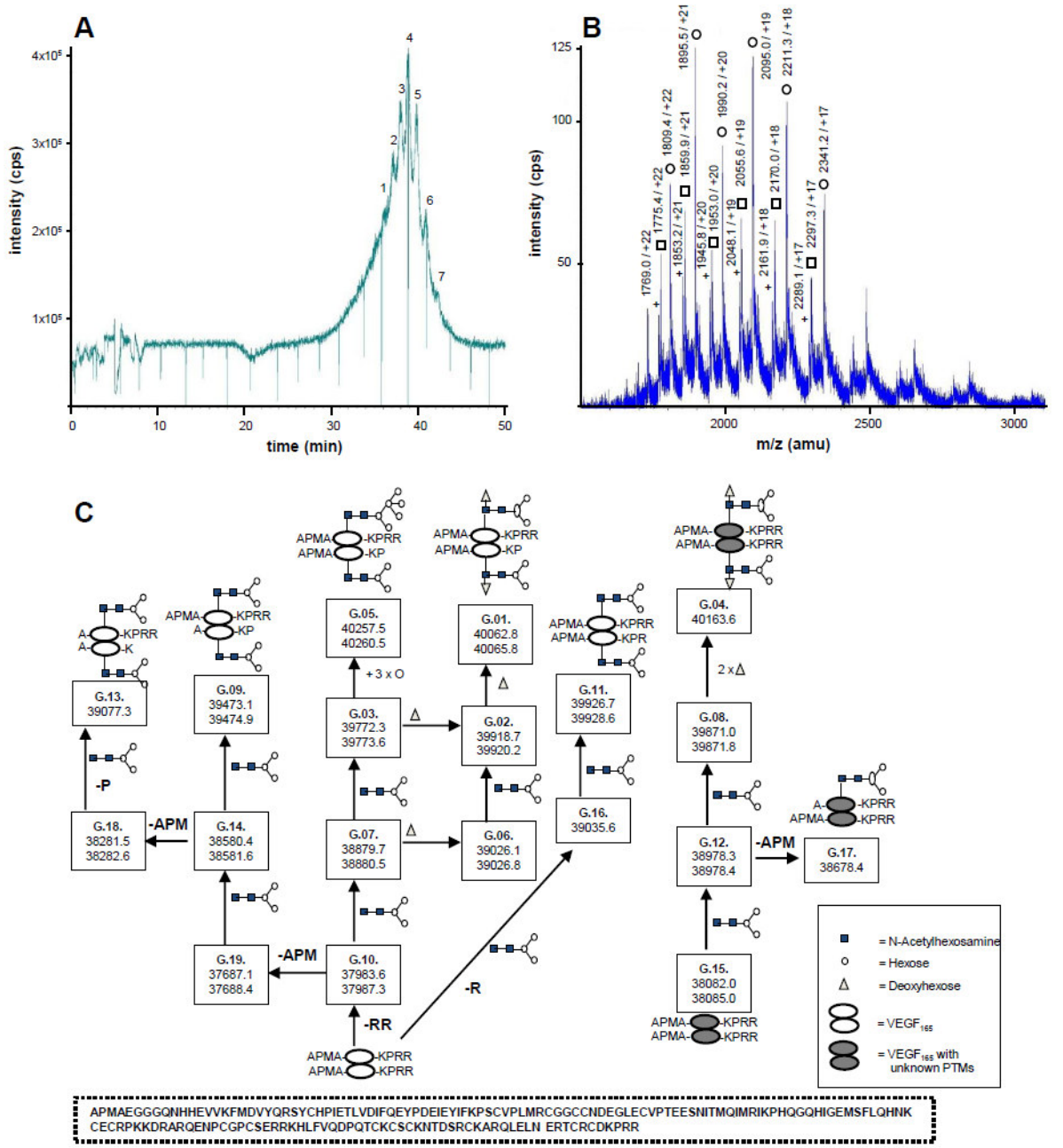

Figure 2.17. (A) CE-TOF-MS analysis of $VEGF_{165}$ on a polycationic polymer coating. (B) MS spectrum of time window 38.5-39.1 min in panel A (peak 4). Different *m/z* series are labeled in the figure (○,□,+,). The *m/z* values and charge numbers are included. (C) Scheme of the different glycoforms observed for $VEGF_{165}$ and relations between them according to the changes in carbohydrate residue and/or in the amino acid sequence of the protein. The amino acid sequence of the protein is shown in the frame [6].

acetylhexosamine (HexNAc) units. In some glycoforms also deoxyhexose units were attached to the biantennary structures. Beside glycoforms, protein variants with a loss of amino acids at the C- and N-terminus were identified giving rise to a total number of 19 different variants of intact $VEGF_{165}$. The CE-MS method could be used to determine the protein in biological samples and it shows potential to become a method for fast quality control when $VEGF_{165}$ becomes a therapeutic drug.

2.2.2 Biopharmaceuticals

Other clinically relevant proteins, such as human growth hormone and hemoglobin-based oxygen carriers, have also been examined by CZE-ESI-MS [7,40-42]. Human growth hormone (hGH) has been analyzed both on a negatively-charged coating using a medium BGE [7,40] and on bare fused-silica capillaries with a low-pH BGE [41]. Catai *et al*. focused on the repeatable and highly efficient separation of hGH and its degradation products [7,40]. After careful optimization of CE and MS parameters, stressed standard solutions and expired pharmaceutical formulations were analyzed. In heat stressed samples, potentially deamidated and dideamidated forms of the protein were observed. Expired formulations also showed the presence of possible deamidation products. However, the resolution of the ion trap (IT) mass spectrometer was not sufficient to unambiguously assign these modifications. On the other hand, CZE-ESI-IT-MS allowed the identification of oxidation products in the pharmaceutical formulation. Staub *et al*. developed a method to distinguish natural hGH from recombinant hGH [41]. A low-pH BGE containing acetonitrile in combination with a bare fused-silica capillary was used to separate the two protein variants within 6 min. After optimization of ESI-TOF-MS interfacing parameters, the method was applied on two samples of unknown origin. In both samples the presence of recombinant hGH could be verified. In addition, in one sample the presence of an oxidized recombinant hGH was observed. Staub *et al*. also developed a CE-ESI-TOF-MS method to analyze hemoglobin-based oxygen carriers (HBOCs) [42]. To prevent adsorption of the proteins to the fused-silica capillary wall a high-pH BGE was used. Hemoglobin and a commercial HBOC were baseline separated under these CE conditions, while with ESI-TOF-MS the proteins were identified. In the gas phase, hemoglobin and the HBOC dissociated into their α- and β-subunits. As the HBOC subunits had a different mass with respect to those of hemoglobin, both proteins could be discriminated from each other. The method was applied to plasma samples enriched with a HBOC, successfully indentifying the protein.

2.2.3 Protein-ligand complexes

CZE-ESI-MS was also used to measure protein-ligand interactions. Fermas *et al*. hyphenated frontal analysis capillary electrophoresis (FACE) with ESI-MS for the analysis of the complex between antithrombin and a sulfated pentasaccharide [8]. A continuous electrokinetic injection was used to introduce the preincubated sample into the CE capillary. As the pentasaccharide is strongly anionic, the complex was more negatively-charged than the free protein thereby allowing the separation of both species (Figure 2.18A and 2.18B). The FACE-MS method was compared to CZE-ESI-MS and it was demonstrated that a higher sensitivity was obtained with FACE-MS, most likely due to the continuous injection. Protein denaturation was observed when a sheath liquid containing water-acetonitrile-formic acid was applied and the noncovalent complex dissociated. Therefore, ammonium acetate (pH 6.5) was used as a non-denaturing sheath liquid. Under these conditions, good quality mass spectra

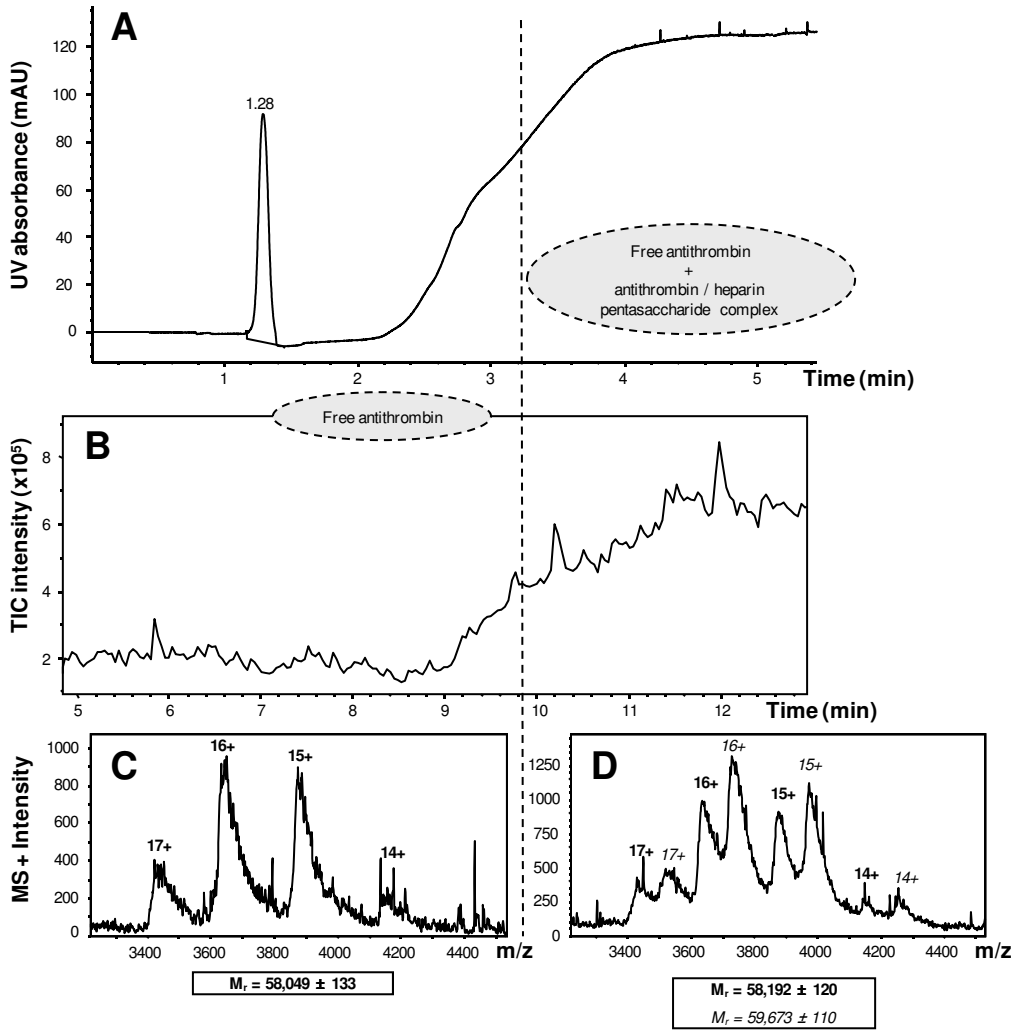

Figure 2.18. (A) UV absorbance electropherogram and (B) TIE of the FACE-ESI-MS analysis of the antithrombin-pentasaccharide interaction. Mass spectra integrated over (C) 9-10 min and (D) 10-12 min time interval show the presence of free antihrombin and the antithrombin-pentasaccharide complex. Antithrombin molecular mass is indicated bold, the molecular mass of the antithrombin-pentasaccharide complex italic [8].

were obtained that allowed indentifying both the nonbound protein and the complex (Figure 2.18C and 2.18D). A similar approach was used to get an insight in the dimerization process of stromal cell-derived factor-1 [43]. It was shown that in the absence of sulfated oligosaccharides, the protein is mostly monomeric in solution. However, dimer formation was observed upon interaction with heparin-sulfated oligosaccharides. Hoffmann and Martin developed a CE-MS method that allowed the analysis of α-chymotrypsin and a chymotrypsin-chymostatin complex under MS-compatible conditions [44]. A poly(vinyl alcohol) coating was applied to prevent protein-wall interactions. An ammonium formate BGE (pH 4.0) allowed the most efficient separation of chymotrypsin and the chymotrypsin-chymostatin complex. A sheath liquid containing water-isopropanol-formic acid (84/15/1, v/v/v) provided the highest protein signals, while maintaining the complex intact. IT-MS/MS enabled the selective release of chymostatin from the enzyme complex. The excitation voltage in which

50% of the chymotrypsin-chymostatin complex dissociated was defined as the binding strength of the complex. Under these conditions other protein-ligand complexes were analyzed and a relative comparison of ligand binding strengths was made based on MS/MS signal areas.

2.2.4 Biomarkers

The combination of CZE with ESI-MS for low molecular weight protein and, especially, polypeptide analysis has also been used in the field of biomarker discovery. The group of Mischak and Schiffer screened both human [45-56] and rat [57,58] urine in order to identify possible biomarkers. Notably, only the fraction of proteins and polypeptides with molecular weights below 20 kDa was analyzed. Vitually for all studies the same CE method employing a low-pH formic acid BGE containing acetonitrile and a bare fused-silica capillary was used. No analytical method development was reported. The CZE-ESI-MS data were presented by plotting migration times of the observed constituents against their deconvoluted molecular masses. Mass lists of the compounds present were derived and the lists of diseased

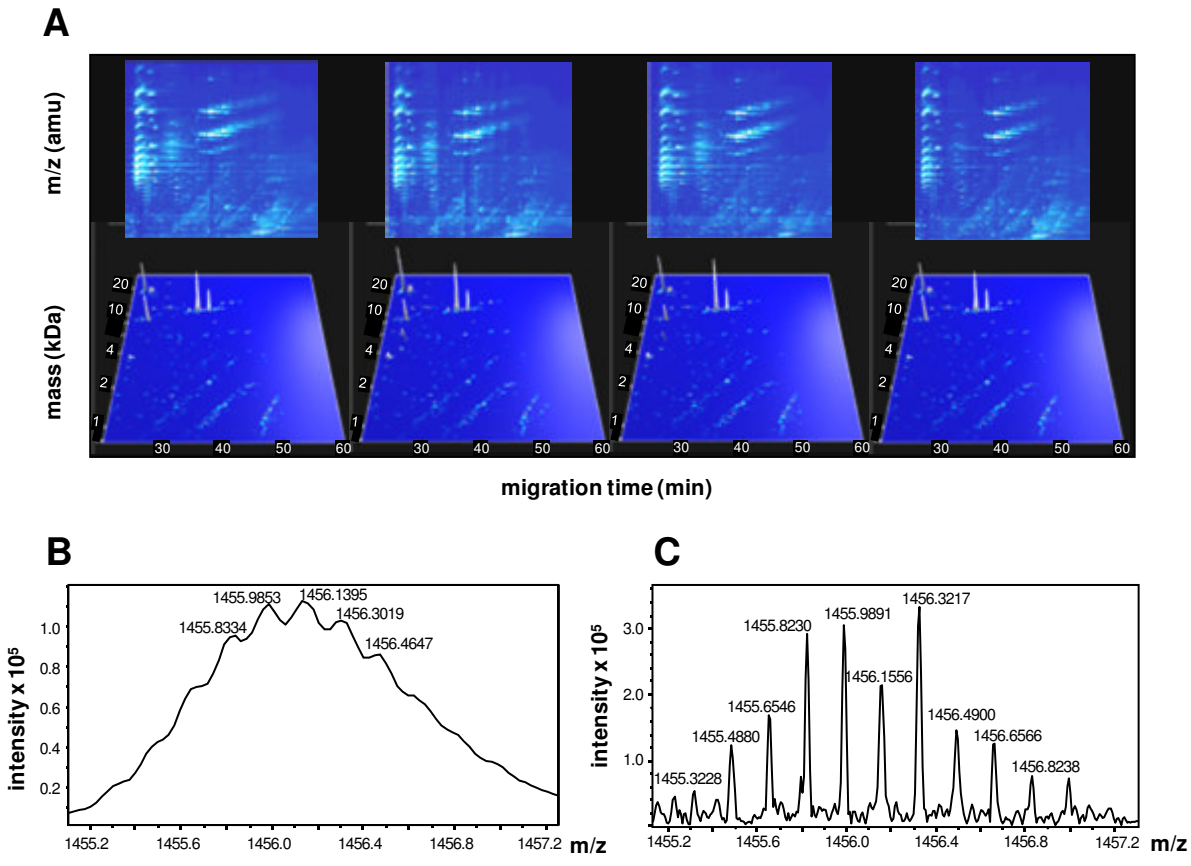

Figure 2.19. (A) Electropherograms obtained for 4 out of 19 consecutive CE-TOF-MS measurements of a single urine sample. The *m/z* values of the 2-D raw data plots (upper panel) and the molecular weight (logarithmic scale) of the deconvoluted 3-D plots (lower panel) on the y-axis are plotted against CE migration time on the x-axis. Isotopic resolution of a sixfold positively charged peptide with a molecular mass of 8725.90 Da as measured with (B) TOF-MS and (C) FT-ICR-MS [57].

and healthy persons were compared. Differences between diseased and healthy indicated the presence of potential biomarkers. Samples of patients suffering from ureteropelvic junction obstruction [45], cancer [46,56], vasculitis [47], coronary artery diseases [48,49,54], kidney diseases [50,51,55], lithium-induced nephropathy [52], graft-versus-host disease [53] and diabetes [54] were investigated. Known or possible new biomarkers were found with the CE-MS method described above. To identify the putative biomarkers MS/MS experiments were also performed. However, identification of all the observed up or down regulated compounds within one study was not possible. A variety of different proteins were marked as potential biomarkers, however, with most diseases different types of collagen appeared to be either up or down regulated. In most cases a TOF mass spectrometer was used. As an example, using CE-TOF-MS for the analysis of rat urine allowed the reproducible determination of on average 1300 polypeptides with a molecular mass between 1 and 20 kDa (Figure 2.19A). They also investigated the use CE in combination with Fourier transform ion cyclotron resonance (FT-ICR) MS in order to obtain very high mass accuracy, especially for the analysis of high molecular mass polypeptides which show unresolved isotopic distributions with TOF-MS [57]. Although the mass resolution of the FT-ICR-MS significantly helped identifying high molecular mass polypeptides (Figure 2.19B and 2.19C), its 20 to 50 fold higher detection limit decreased the number of traceable polypeptides with approximately a factor of 15. Still, 100 rat urinary polypeptides could be defined with high accuracy. The high accuracy masses of these polypeptides were used to calibrate the CE-TOF-MS mass data sets before further data analysis, resulting in an improved analytical precision.

2.2.5 Food proteins

CZE-ESI-MS has also found its application in the analysis of food products [59-61]. Cifuentes *et al*. developed a CE-MS method to analyze zein protein fractions from maize [59]. A positively-charged coating in combination with a BGE containing acetonitrile, isopropanol water and formic acid was applied to obtain efficient protein separations (Figure 2.20A). In order to obtain the highest signal-to-noise ratios for the proteins, an optimization of the sheath liquid and ESI-MS parameters was performed. Good quality mass spectra were obtained (Figure 2.20B), that allowed identification of the zein proteins. The usefulness of the CE-MS method was investigated by comparing the zein-protein fingerprints of various maize lines cultivated under the same conditions. Up to 13 different proteins could be separated and identified in each maize line. Also a comparison between IT-MS with TOF-MS for the analysis of zein protein mixtures was made [60]. It was shown that CE-ESI-IT-MS provides MS spectra of intact proteins with less background signals, but CE-ESI-TOF-MS allows the identification of a higher number of proteins from complex matrices. Muller *et al*. described the development of a CE-MS method aimed at the detection and quantification of bovine milk in either ovine or caprine milk samples by the analysis of the whey proteins α-lactalbumin and

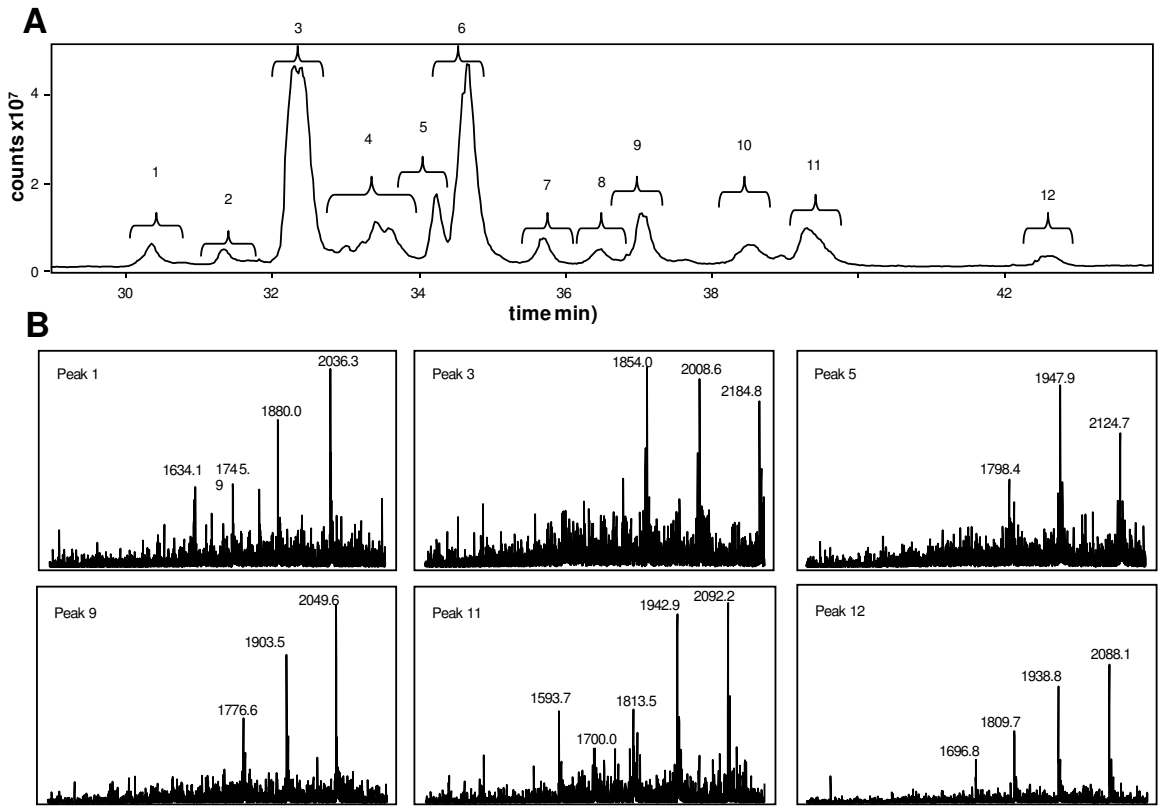

Figure 2.20. (A) Optimized CZE-ESI-MS separation of zein proteins using a BGE containing acetonitrile, isopropanol, water and formic acid (40/20/38/2, v/v/v/v). (B) MS spectra of six selected peaks depicted in panel A [59].

various β-lactoglobulins [61]. A 1 M formic acid BGE was used to minimize protein adsorption to the silica capillary. Under these conditions, α-lactalbumin and β-lactoglobulin from bovine milk had a different electrophoretic mobility than the proteins originating from ovine and caprine milk. Clear mass spectra were obtained with a water-methanol-formic acid sheath liquid, showing that the molecular weight of the proteins from different origin were not the same. Therefore, the combination of electrophoretic mobility and molecular weight allowed a reliable evaluation of milk adulteration. Concentrations below 5% bovine milk in caprine milk could be determined reliably, which is similar to lowest measurable adulteration limits reported with CE-UV [62].

3. CE-MALDI-MS

In the past years, CE-MALDI-MS was not frequently applied for the analysis of intact proteins. Research was mainly focused on the coupling, development and optimization (Table 2.8; p. 89). Two major developments were reported in the field of CZE-MALDI-MS of intact proteins. Yassine *et al*. developed an off-line coupling of CE with microwave-assisted acid hydrolysis (MAAH)-MALDI-MS for protein identification and characterization [63]. Preparative scale protein separations in surfactant-coated capillaries enabled collection of purified cytochrome *c*. The protein was mixed with hydrochloric acid and subsequently

digested in a microwave. The digest was spotted on a MALDI target and the protein could be sequenced. They observed that BGE concentrations above 40 to 60 mM decreased peptide signals in MALDI-MS up to a factor four. After optimization of the BGE concentration, a sequence coverage up to 95% was obtained for cytochrome *c*. Jacksen *et al*. presented a new technique for off-line hyphenation between CZE and MALDI-MS [64]. A silicon chip comprising an open microchannel was integrated in between two fused-silica capillaries. CZE separations were stopped as soon as all separated components migrated through the microchannel. MALDI matrix was manually added to the chip to allow crystallization in the channel and, subsequently, the chip was placed in a MALDI-TOF mass spectrometer. This system allowed the separation of lysozyme and cytochrome *c* within 11 min and good quality mass spectra were obtained for both proteins.

Research within the field of CIEF-MALDI-MS for intact proteins has primarily been focused on the optimization of separation and spotting conditions [65-67]. Lechner *et al*. used a home-made deposition tip for the automated sample deposition from the separation capillary onto a MALDI target [65]. Various spotting conditions were optimized after which a five-protein test sample was analyzed. All five proteins were collected in different spots on the MALDI plate, allowing accurate p*I* determinations. The ionization yield was sufficiently high to obtain clear mass spectra and molecular masses with accuracies within 2500 ppm. Weiss *et al*. proposed the hardware and protocol necessary to make syringe pump mobilization compatible for CIEF-MALDI-MS interfacing [67]. One of the major problems in this mobilization approach is the formation of bubbles, but following the protocol, reproducible elution times of markers and accurate p*I* determination of intact proteins was demonstrated. Silvertand *et al*. extensively describes the hyphenation of CIEF and MALDI-TOF MS via a spotting device [66]. MS electropherograms were reconstructed by plotting the intensities of the *m/z* values corresponding to the proteins versus migration time (related to spot number). Focusing and spotting times were optimized using these reconstructed MS electropherograms as well as the UV electropherograms. The applicability of the system is demonstrated by the

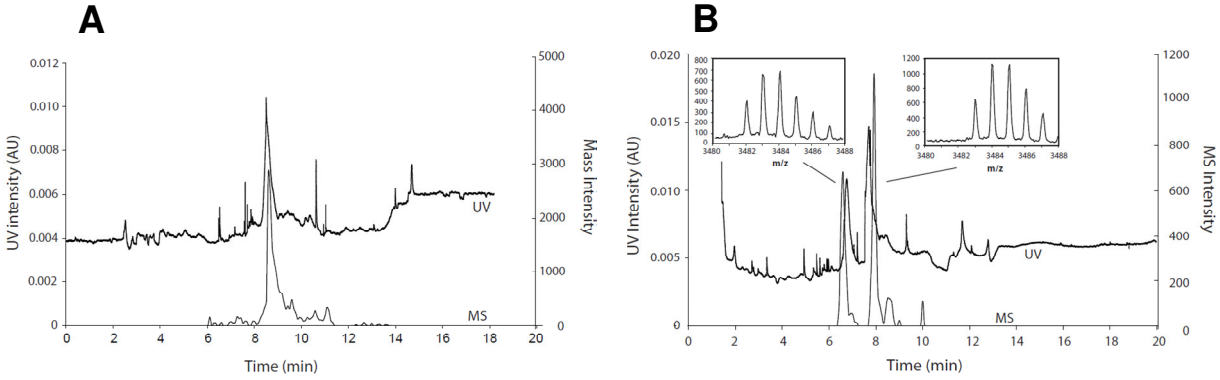

Figure 2.21. Reconstructed MS electropherograms (thin trace) and UV electropherograms (bold trace) of (A) intact glucagon and (B) degraded glucagon. Mass spectra of glucagon and the degradation product are shown in the insets [66].

analysis of the biopharmaceutical glucagon that was incubated with hydrochloric acid for 2 weeks (Figure 2.21). Both glucagon and a single degradation product were baseline separated. The degradation product was identified as a single deamidation based on the observed pI-shift and mass difference.

4. CE-ICP-MS

CE-ICP-MS is limited to metal-containing and metal-binding proteins. Meanwhile, it has reached a stage of maturity and several applications were reported in the last years (Table 2.9; p. 90). Some reports on the binding of metallodrugs to human serum albumin (HSA) and transferrin (Tf) as studied by CE-ICP-MS have been published [68-73]. The binding of ligands to these proteins is very important as, in general, HSA and Tf are the main binding partners of various drugs. Affinities of anticancer drugs [68-72] and antidiabetic complexes [73] towards both protein were monitored. The CE analysis of HSA and Tf can be carried out using a bare fused-silica capillary with a buffer at physiological pH (7.4). Groessl *et al.* showed that the separation between the two proteins could be improved by the introduction of

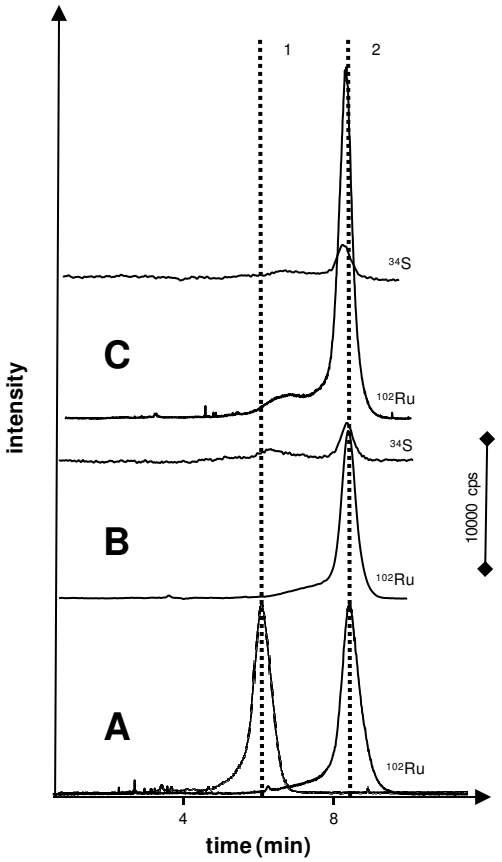

Figure 2.22. CZE-ICP-MS analysis of samples containing either Tf or HSA that were in vitro treated with a Ru-based anticancer drug (A). CE-ICP-MS analysis of a clinical sample from a patient on day 2 (B) and on day 18 (C) after first administration of a Ru-based anticancer drug. For each clinical sample, both the Ru- and S-content as monitored with ICP-MS are depicted. The S-content is an indication of the presence of a protein, while the Ru-content indicates the presence of the drug. Clearly, the drug binds specifically to HSA. Peak identification: 1, Tf adduct; 2, HSA adduct [71].

a positively-charged Polybrene (PB) coating in combination with a low-pH BGE [71]. They demonstrated that the binding of a Ru(III)-based drug with both proteins could be monitored under these conditions (Figure 2.22A). The binding of the drug in patients (Figure 2.22B and 2.22C) was found to be in agreement with the behavior of the drug in *in vitro* tests. In a subsequent publication, they observed that Ga(III)-based drug binding with apoTf was compromised when BGEs with a pH below 7.0 were used [69]. A carbonate-based BGE at pH 7.4 gave most reliable binding results while still obtaining a partial separation of free Ga, Ga-Tf and Ga-HSA. Carbonate was required in the BGE as a synergistic anion to stabilize the noncovalent binding of the drug to Tf. It was assumed that without carbonate in the BGE the release of Ga(III) species from the protein was induced. Similar behavior was described by Bytzek at al. [73] for a Zn(II)-based antidiabetic drug. With a Tris BGE, the CE-ICP-MS resulted in a peak representing 20% drug binding, whereas with a carbonate-containing BGE a peak representing 50% drug binding was observed. Interestingly, others do not mention this behavior when a phosphate BGE (pH 7.4) was used to study the binding of a Ga(III)-based drug with apoTf [72].

5. Conclusions

As highlighted within this review, CE-MS is an interesting tool for the analysis of intact proteins. It allows both efficient separations and detailed characterization of proteins and their impurities. The combination of CE with ESI-MS has reached maturity, which is reflected in a considerable amount of applications. Still, research in improved CE-ESI-MS interfacing is ongoing in order to further push the limits for CE-MS in terms of analytical performance. To be suited for a variety of applications, like biopharmaceutical quality control or biomarker discovery, CE-MS systems that allow the determination of low protein concentrations are required. Although coupling CIEF and chip-based electrophoresis to ESI-MS is not straightforward, the articles published over the past years clearly demonstrate that these techniques are getting up to levels with established CE-MS methodologies.

There are still some interesting application fields for CE-ESI-MS of intact proteins that have hardly been explored. For example, it might be attractive to study protein unfolding states by CE-ESI-MS. Proteins can be separated under mild conditions, whereas ESI-MS can provide information on the protein conformational state in solution [74]. Furthermore, the CE-MS analysis of intact monoclonal antibodies (mAbs), which represent the most significant part of today's biopharmaceuticals, has been hardly explored yet. Many modifications, such deamidation, glycosylation, pyroglutamic acid formation, and peptide bond cleavage [75], may affect the quality and properties of mAbs. As these modifications may involve a change in net charge of the protein, CE would be a very suitable tool to separate these species. ESI-MS can yield useful information on molecular mass [76]. The combination of these techniques would definitely contribute to the characterization of these proteins.

Recent research in CE-MALDI-MS mainly focused on further development of the technique, but the applications still demonstrate its usefulness for intact protein analysis. With TOF analyzers the upper mass limit is virtually unlimited, making the technique well suited for detection of high molecular weight compounds, like mAbs [77]. Efficient CZE or CIEF separations may allow separation of different protein isoforms and, therefore, CE-MALDI-MS might also be an interesting approach for the analysis of mAbs.

Currently, CE-ICP-MS is a common tool for the analysis of metal-containing proteins or to study the binding of metal-containing ligands, like drugs, to proteins. ICP-MS provides an elegant means to reveal metal stochiometry, but no information on protein identity is obtained. Up to now, only CZE was combined with ICP-MS. However, recently it was demonstrated that the on-line coupling of gel electrophoresis with ICP-MS can be used for the determination of protein phosphorylation [78,79] and iron content [80]. Considering this research, it might be interesting to investigate whether CIEF and capillary gel electrophoresis can be combined with ICP-MS. This would significantly enhance the applicability of the technique.

A drawback of analyzing intact proteins with CE-MS might be that an unambiguous assignment of protein modifications is not always possible. To facilitate the identification and position of modifications, bottom-up approaches can be included into on-line CE-MS. Recently, the coupling of enzyme reactors for CE-ESI-MS were introduced [81,82], however, they could as easily be used in combination with either MALDI-MS or ICP-MS . Both an enzyme reactor placed at the end the separation capillary and a reactor in between two separation capillaries, allowing a separation of the proteins in the first capillary and a peptide separation in the second capillary, were discussed. These approaches proved promising for the identification of proteins and protein impurities.

Table 2.7. CE-ESI-MS for intact protein analysis.

Analytes[a]	Matrix	BGE/ampholytes	Capillary coating[b]	Mass analyzer[c]	Remarks	Ref.
capillary zone electrophoresis						
antithrombin	aqueous solution	30 mM ammonium hydrogencarbonate (pH 8.5)	no coating	IT	frontal analysis to measure protein-ligand interaction	[8]
aprotinin, cyt *c*, α-CT,	aqueous solution	75 mM ammonium acetate (pH 6.5)	polyethylene oxide	IT	dynamic coating for on-line CE-MS	[26]
CA, cyt *c*, Hb, tryp	aqueous solution	0.1% acetic acide, 0.1% Polybrene	PB	IT	new interface design	[20]
CA, Hb	red blood cells	1.7 mM ammonium acetate (pH 7.4), 0.1% Polybrene	PB	QTOF	determination Hb and CA in red blood cells	[22]
CAII, hGH, insulin, α-lac	aqueous solution, pharmaceutical formulation	75 mM ammonium formate (pH 8.5)	PB-PVS	IT	optimizing CE-MS conditions and characterizing protein degradation products	[40]
CA, insulin, RNase A, lys	aqueous solution	100 mM ammonium acetate (pH 3.1)	polyethylenimine	TOF	characterization sheathless interface for protein analysis	[21]
cyt *c*, Hb, lys, myo, β-LG, ribosomal proteins	*Escherichia coli* extract	0.1% acetic acid, 33 nM PB	PB	IT	dynamic coating for on-line CE-MS	[25]
cyt *c*, lys, RNase A	aqueous solution	100 mM ammonium acetate (pH 3.1)	polyacrylamide	TQ	new liquid-junction interface design	[19]
cyt *c*, lys, RNase A, α-CT,	aqueous solution	10 mM ammonium acetate (pH 3.0)	M7C4I	TOF	coating characterization	[23]
EPO, NESP	pharmaceutical formulation	2 M acetic acid	acryl amide	IT	glycoform profiling	[36]
Hb	aqueous solution, human plasma	75 mM ammonium formate (pH 9.5)	no coating	TOF	determination of Hb-based oxygen carriers in plasma	[42]
hGH	aqueous solution, pharmaceutical formulation	75 mM ammonium formate (pH 2.5), 20% acetonitrile	no coating	TOF	method optimization for impurity profiling	[41]
hGH	pharmaceutical formulation	75 mM ammonium formate (pH 8.5)	PB-PVS	IT	characterizing protein degradation products	[7]
llama antibody	aqueous solution	175 mM acetic acid (pH 2.7)	PB-DS-PB	TOF	coating characterization	[24]
polypeptides, small proteins	human urine	0.5% formic acid, 30% methanol	no coating	TOF	screening for ureteropelvic junction obstruction in infants	[45]
polypeptides, small proteins	human urine	250 mM formic acid, 20% acetonitrile	no coating	TOF/FT-ICR	biomarker discovery cancer	[46, 56]

Table 2.7. Continued

polypeptides, small proteins	human urine	250 mM formic acid, 20% acetonitrile	no coating	TOF	biomarker discovery vasculitis	[47]
polypeptides, small proteins	human urine	250 mM formic acid, 20% acetonitrile	no coating	TOF	biomarker discovery coronary artery diseases	[48, 49]
polypeptides, small proteins	human urine	250 mM formic acid, 20% acetonitrile	no coating	TOF	biomarker discovery kidney diseases	[50, 51, 55]
polypeptides, small proteins	human urine	250 mM formic acid, 20% acetonitrile	no coating	TOF	biomarker discovery lithium-induced nephratology	[52]
polypeptides, small proteins	human urine	250 mM formic acid, 20% acetonitrile	no coating	TOF	proteomic patterning graft-versus-host disease	[53]
polypeptides, small proteins	human urine	250 mM formic acid, 20% acetonitrile	no coating	TOF	biomarker discovery various diseases	[54]
polypeptides, small proteins	rat urine	250 mM formic acid, 20% acetonitrile	no coating	TOF/FT-ICR	peptidomic analysis; comparing different MS analyzers	[57]
polypeptides, small proteins	rat urine	250 mM formic acid, 20% acetonitrile	no coating	TOF	proteomic patterning drug-induced nephrotoxicity	[58]
rabbit liver MTs	aqueous solution	50 mM acetic and formic acid (pH 2.3)	no coating	IT	modeling protein migration behavior	[83]
rabbit liver MTs	aqueous solution	50 mM acetic and formic acid (pH 2.3)	no coating	IT	classification and characterization sub-isoforms	[84]
r-αHCG	aqueous solution	2% acetic acid (pH 2.5)	poly(vinyl alcohol)	FT-ICR	glycoform profiling	[37]
stromal cell-derived factor-1	aqueous solution	75 mM ammonium acetate (pH 6.5)	polyethylene oxide	IT	frontal analysis to measure protein-ligand interaction	[43]
superoxide dismutase	aqueous solution	different compositions	no coating	IT	characterizing protein structure	[85]
Tf	human serum	25 mM ammonium acetate (pH 8.5)	PB-DS	TOF	glycoform profiling	[38]
$VEGF_{165}$	aqueous solution	60 mM formic acid	poly-LA 313	TOF	glycoform profiling	[6]
zein-proteins	maize extracts	acetonitrile / isopropanol / formic acid / water (40/20/2/38)	EPyM/DMA	TOF/IT	comparing mass analyzers for intact protein detection	[60]
zein-proteins	maize extracts	acetonitrile / isopropanol / formic acid / water (40/20/2/38)	EPyM/DMA	IT	comparing strains of maize	[59]

Table 2.7. Continued

α-1-acid glycoprotein	human serum	1 M acetic acid	EPyM/DMA	QTOF	glycoform profiling	[39]
α-CT	aqueous solution	47.2 mM ammonium formate (pH 4.0)	poly(vinyl alcohol)	QIT	characterizing protein-ligand interactions	[44]
α-lac, β-LGA, β-LGB	milk extracts	1 M formic acid (pH 1.9)	no coating	IT	monitoring milk adulteration	[61]
capillary isoelectric focusing						
CAII, myo, RNase A, TI, α-CT, β-LGA	aqueous solution	Beckman ampholytes (p*I* 3-10)	no coating	SQ	optimization for online cIEF-ESI-MS. Glycerol added to BGE to suppress EOF.	[29]
various	*Chlorobium tepidum* lysate	Beckman ampholytes (p*I* 3-10)	polyacrylamide	QTOF	cIEF-RPLC-MS for ampholyte removal and additional separation; LC coupled to MS	[31]
chip-based electrophoresis						
cyt *c*, myo, RNase A	aqueous solution	methanol / 0.2% acetic acic (50/50)	polyE-323	QTOF	characterizing chip CE-MS	[33]
Hb	single cells	methanol / 2% acetic acic (50/50)	polyE-323 coating	QTOF	single cell analysis	[34]
Myo	aqueous solution	methanol / 2% acetic acid / 1-methoxy-2-propyl acetate (50/45/5)	1-methoxy-2-propyl acetate	TQ	dynamic coating for on-line CE-MS	[32]

a. CAII, carbonic anhydrase; cyt *c*, cytochrome *c*; EPO, erythropoietin; Hb, hemoglobin; hGH, human growth hormone; lys, lysozyme; MT, metallothionein; myo, myoglobin; NESP, novel erythropoietin stimulating protein; RNase A, ribonuclease A; r-αHCG, recombinant α subunit of human chorionic gonadotrophin; Tf, transferrin; TI, trypsin inhibitor; tryp, trypsinogen; $VEGF_{165}$, vascular endothelial growth factor 165; α-CT, α-chymotrypsinogen; α-lac, α-lactalbumin; β-LGA, β-lactoglobulin A; β-LGB, β-lactoglobulin B.

b. DS, dextran sulfate; EPyM/DMA, ethylpyrrolidine methacrylate-N,N-dimethylacrylamide; M7C4I, 1-(4-iodobutyl) 4-aza-1-azoniabicyclo(2,2,2) octane iodide; PB, Polybrene; PVS, poly (vinylsulfonic acid).

c. FT-ICR, Fourier transform ion cyclotron resonance; IT, iontrap; QIT, quadrupole iontrap; QTOF, quadrupole time-of-flight; SQ, single quadrupole; TOF, time-of-flight; TQ, triple quadrupole.

Remark: for all the chip-based separations CZE was used to analyze the proteins.

Table 2.8. CE-MALDI-MS for intact protein analysis.

Analytes[a]	Sample matrix	BGE/ampholytes	Capillary coating	Interface	MALDI matrix[b]	Remarks	Ref.
capillary zone electrophoresis							
cyt *c*	aqueous solution	100 mM bis-tris phosphate (pH 7.0)	cationic surfactants	offline fraction collection in vial	CHCA	microwave-assisted acid hydrolysis on target for bottom-up protein analysis	[63]
cyt *c*, lys	aqueous solution	50 mM phosphate (pH 4.0)	didodecyl dimethyl ammonium bromide	offline open microchannel	DHB	separation partially takes place in an open channel that is on a MALDI target plate.	[64]
cyt *c*, Hb, HSA, lys, RNase A, tryp, α-lac	aqueous solution	250 mM acetic acid (pH 3.0)	poly(vinyl alcohol)	offine sheath-liquid fraction collector in vial	sinapinic acid		[86]
capillary isoelectric focusing							
BSA, ins, lys, myo, ubi	aqueous solution	2.5% Servalyte (p*I* 3-10)	poly(vinyl alcohol)	offline sheath flow collector on target	THAP/diammonium hydrogen citrate	characterization automatic sample deposition	[65]
CAII, glu, myo, RNase A, α-lac, β-LGA, β-LGB	aqueous solution, pharmaceutical formulation	1% Pharmalyte (p*I* 3-10)	eCAP neutral	offline sheath flow collector on target	sinapinic acid	optimization separation and spotting conditions	[66]
myo, β-LGA, β-LGB	aqueous solution	2% BioChemika ampholyte (p*I* 5-8)	used, but not specified	offline sheath flow collector on target	sinapinic acid	optimization separation and spotting conditions	[67]

a. BSA, bovine serum albumin; CAII, carbonic andyrase II; cyt *c*, cytochrome *c*; glu, glucagon; Hb, hemoglobin; HSA, human serum albumin; ins, insulin; lys, lysozyme; myo, myoglobin; RNase A, ribonuclease A; tryp, trypsinogen; ubi, ubiquitin; α-lac = α-lactalbumin; β-LGA, β-lactoglobulin A; β-LGB, β-lactoglobulin B.

b. CHCA, α-cyano-4-hydroxycinnamic acid; DHB, 2,5-dihydroxybenzoic acid; THAP, trihydroxyacetophenone monohydrate.

Remark: in all cases a time-of-flight mass spectrometer was used.

Table 2.9. CE-ICP-MS for intact protein analysis

Analytes[a]	Sample matrix	Elements	BGE[b]	Remarks	Ref.
HSA	human plasma	Pt	10 mM phosphate (pH 7.4)	interface comparison for Pt determination in plasma	[68]
HSA, Tf	aqueous solution	Ga, S, Fe	10 mM ammonium bicarbonate (pH 7.4)	assaying in vitro metallodrug metabolism	[69]
HSA, Tf	aqueous solution	Ru	10 mM phosphate (pH 7.4)	assaying in vitro metallodrug metabolism	[70]
HSA, Tf	aqueous solution, human plasma and serum	Ru, S	50 mM formic acid (pH 2.7)	PB coating, protein-drug binding	[71]
HSA, Tf	artificial intestine juice, human serum	Ga, Fe	10 mM phosphate, 10 mM NaCl, 40 mM HEPES (pH 7.4)	assaying in vitro metallodrug metabolism	[72]
HSA, Tf	human serum	Zn, S	10 mM carbonate (pH 7.4) and 20 mM TRIS	protein-drug binding	[73]

a. HSA, human serum albumin; Tf, transferrin.

b. HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TRIS, 2-Amino-2-hydroxymethyl-propane-1,3-diol.

Remark: in all cases CZE was used to separate the proteins and a single quadruple mass analyzer was used to detect the elements of interest. Furthermore, a microconcentric nebulizer was used in all research to interface CZE with ICP-MS.

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Chapter 3

Effectiveness of charged noncovalent polymer coatings against protein adsorption to silica surfaces studied by evanescent-wave cavity ring-down spectroscopy and capillary electrophoresis

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Abstract

Protein adsorption to silica surfaces is a notorious problem in analytical separations. Evanescent-wave cavity ring-down spectroscopy (EW-CRDS) and capillary electrophoresis (CE) were employed to investigate the capability of positively-charged polymer coatings to minimize the adsorption of basic proteins. Adsorption of cytochrome *c* (cyt *c*) to silica coated with a single layer of Polybrene (PB), or a triple layer of PB, dextran sulfate (DS) and PB was studied and compared to bare silica. Direct analysis of silica surfaces by EW-CRDS revealed that both coatings effectively reduce irreversible protein adsorption. Significant adsorption was observed only for protein concentrations above 400 μM whereas the PB-DS-PB coating was shown to be most effective and stable. CE analyses of cyt *c* were performed with and without the respective coatings applied to the fused-silica capillary wall. Monitoring of the electroosmotic flow and protein peak areas indicated a strong reduction of irreversible protein adsorption by the positively-charged coatings. Determination of the electrophoretic mobility and peak width of cyt *c* revealed reversible protein adsorption to the PB coating. It is concluded that the combination of results from EW-CRDS and CE provides highly useful information on the adsorptive characteristics of bare and coated silica surfaces towards basic proteins.

1. Introduction

Developments in the fields of protein chemistry, biopharmaceuticals and biotechnology have led to an increasing demand for sensitive and selective analytical tools for the determination of intact proteins. Especially separation techniques that allow analysis of proteins under mild conditions without the need for, for example, organic solvents or very high salt concentrations, gain growing interest. However, when separating proteins by, for example, capillary electrophoresis (CE) or microfluidic systems the tendency of protein molecules to adsorb to the internal walls of the glass or (fused-)silica flow channels is a major problem. This is particularly true for basic (*i.e.*, positively charged) proteins. As a result, separation efficiencies deteriorate, unless the surfaces are extensively reconditioned after each run. To prevent these protein-wall interactions, coating of the internal surfaces with agents that minimize protein adsorption is a common strategy [1-3].

To establish the effectiveness of such coatings, methods are required that allow determination of reversible and irreversible adsorption. In CE, parameters such as protein electrophoretic mobility, peak asymmetry and plate number can be used to assess reversible adsorption. In addition, changes in peak height or area between runs and in electroosmotic flow (EOF) may indicate irreversible protein adsorption [2]. Regnier *et al.* [4] proposed a method to probe protein adsorption in CE. They devised a special instrument which allowed protein detection at several positions along the capillary. A decrement of peak area as measured by the successive detectors quantitatively indicated protein adsorption. Righetti and co-workers [5] developed a capillary equilibration procedure to probe the amount of adsorbed protein. The capillary was first completely filled and conditioned with a background electrolyte (BGE) containing fluorescently labeled protein. After equilibration, the capillary was flushed with buffer to remove any unbound protein. The protein retained on the capillary was then eluted with surfactant-containing buffer and subsequently quantified by laser-induced fluorescence detection. The method was used to evaluate the ability of several additives to prevent protein adsorption.

As discussed above, in a CE setting, protein adsorption is normally studied in an indirect fashion, because probing of the actually adsorbed protein molecules at the capillary wall obviously would be very difficult, if not impossible. *In-situ* monitoring of adsorption and desorption processes at surfaces can in principle be achieved using surface-specific spectroscopic methodologies, such as surface plasmon resonance (SPR) and attenuated total reflection (ATR). SPR is based on the measurement of the shift in the plasmon resonance of a thin metal (usually gold) film after adsorption of species to the metal or a layer (*e.g.* a self-assembled monolayer) deposited on the metal. In order to use SPR for protein adsorption studies on silica, a synthetic silica layer should be deposited on the gold SPR surface. Such a system would not be an appropriate model for studying the effectiveness of coatings. Kraning *et al.* [6] recorded polarization-dependent absorption spectra on a single-pass silica ATR prism, which was sufficiently sensitive for the detection of a full monolayer of cytochrome *c.*

However, for detection of lower surface coverage, the absorbance signal needs to be enhanced. In ATR spectroscopy this can be achieved by increasing the optical path-length using a multipass geometry. Still, to get a sufficient number of reflections at the surface, waveguides with dimensions on the order of several centimeters have to be used, necessitating large sample volumes.

Recently, evanescent-wave cavity ring-down spectroscopy (EW-CRDS) was shown to be an effective tool for the probing of surface-specific processes, and its potential for bioanalytical applications was demonstrated [7-9]. CRDS is a very sensitive mode of absorbance spectroscopy based on the detection of the exponential decay of light behind an optically stable cavity after abrupt termination of excitation. Adding an absorber (analyte) in the cavity constitutes an extra loss of light resulting in a shortened 1/e or ring-down time of the exponential decay [10,11]. Although CRDS was first developed for measuring very low gas-phase absorbances, more recently, CRDS applications in the liquid phase have also been reported, as reviewed by Van der Sneppen and co-workers [11]. In EW-CRDS, one of the reflections in the cavity is a total internal reflection (TIR) event. Only the evanescent wave associated with the TIR is being used to probe the sample. EW-CRDS combines the excellent sensitivity of CRDS with the surface specificity of EW techniques and, therefore, in principle is suited for studying protein adsorption to silica surfaces [7-9]. An adsorbed protein of which the UV-vis absorption spectrum overlaps with the applied laser wavelength can be detected through a decrease in the ring-down time, whereas unbound protein in the bulk solution will hardly contribute to the EW-CRDS signal.

EW-CRDS studies of compounds on coated prisms have been reported [7,12-16]. The applied surface coatings mainly served to bind the compounds of interest [7,12-15] and not to prevent adsorption. The coatings were either covalently [7,13,16] or noncovalently [12,14,15] attached to the silica surface. Covalent coatings can be very effective, however, their production can be tedious and time-consuming, and may not be reproducible. Noncovalently attached coatings offer a more practical solution, as they can be made simply by flushing the solution of the proper coating agent along the silica surface. This type of coating is generally based on the physical adsorption due to electrostatic interaction between the coating agent and the silica surface. Such a coating procedure is relatively fast and can be easily repeated. An interesting and highly flexible approach, as introduced by Katayama *et al*. for CE [17], is the use of successive multiple charged-polymer layers to construct stable coatings. Powell *et al.* [12] applied EW-CRDS to study the pH-dependent adsorption of a ruthenium-based dye to a noncovalent bilayer coating of poly-L-lysine and poly-L-glutamic acid.

In the present study, EW-CRDS is used to evaluate the effectiveness of charged noncovalent polymer coatings in minimizing adsorption of basic proteins. The coatings were applied to the surface of a fused-silica EW-CRDS prism. The studied coatings were a single layer of Polybrene (PB) and a triple layer composed of PB, dextran sulfate (DS) and PB (PB-DS-PB) [18]. These positively charged coatings were prepared by flushing the silica surface

with PB, or successively with PB and DS solutions. Subsequently, the respective coatings were exposed to solutions of cytochrome *c* (cyt *c*) in sodium phosphate buffer (pH 7.4). Cyt *c* has a p*I* of 10.5 and is consequently positively charged at medium pH. It is chosen as a test compound as it tends to adsorb strongly to bare silica [4,6,16] and features a strong absorption in the visible range. Cyt *c* adsorption to bare and coated silica prisms was measured directly with EW-CRDS at 538 nm which overlaps with the strongest Q-band transition of cyt *c* and for which wavelength appropriate mirrors were available in our laboratory. Cyt *c* was also analyzed by CE using a fused-silica capillary without and with the respective coatings in combination with a BGE of sodium phosphate (pH 7.4). The CE results in terms of magnitude of the EOF, peak area, electrophoretic mobility and peak width are interpreted with respect to protein adsorption and discussed in conjunction with the EW-CRDS measurements.

2. Experimental

2.1. Chemicals

Potassium hydroxide, Polybrene (hexadimethrine bromide, PB) and dextran sulfate (DS) sodium salt were purchased from Sigma-Aldrich (Steinheim, Germany). Sulfuric acid, acetonitrile and potassium dihydrogen phosphate were obtained from Merck (Darmstadt, Germany). Formamide was obtained from Fluka (Steinheim, Germany) and used as an EOF marker. A 10 mM phosphate buffer (pH 7.4) was prepared by dissolving 0.136 g of potassium dihydrogen phosphate in 100 mL of deionized water and adjusting the pH with potassium hydroxide. Solutions of 10% (w/v) PB and 3% (w/v) DS were prepared in deionized water. The solutions were filtered over a 0.45 μm filter type HA (Millipore, Molsheim, France) prior to use.

For the EW-CRDS measurements, a stock solution of 1 mM (12.5 mg/mL) bovine heart cytochrome *c* (Sigma-Aldrich) was made in 10 mM potassium phosphate buffer and further diluted to the required concentration with the phosphate buffer. For the CE experiments, a stock solution of 80 μM (1 mg/mL) cyt *c* was prepared in deionized water, and diluted in deionized water containing 0.25% (v/v) formamide (EOF marker) to a final cyt *c* concentration of 16 μM.

2.2 EW-CRDS system

The set-up was similar to the one used in a previous study [16] and is shown in Figure 3.1. A crucial difference was that a 10-Hz Nd:YAG-pumped pulsed dye laser system was used; as a consequence, in this study the light was s-polarized, as opposed to p-polarized. An optical cavity was constructed using mirrors with a reflectivity $R \geq 99.996\%$ at 532 nm, 50 mm radius of curvature from REO Inc. (Boulder, CO, USA), in combination with a 70-degrees Dove prism at normal incidence. This way, reflections at the intracavity surfaces do not lead to losses, but are maintained within the cavity. Entrance and exit faces of the prism

were both polished to a flatness of λ/10 at 632.8 nm in order to minimize losses at these faces, whereas the TIR face was polished to a flatness of λ/2. Excitation of the cavity at 538 nm was performed with pulses (0.5 – 1 mJ) from a Quanta-Ray PDL-3 pulsed dye laser with coumarin 152, pumped by the 355-nm output (third harmonic) of a Quanta-Ray Nd:YAG laser (Spectra-Physics, Mountain View, CA, USA) at a repetition rate of 10 Hz and a pulse duration of 5 ns. In these experiments, the EW probes the sample layer closest to the surface approximately 100 times. After each laser pulse the light recorded by a photomultiplier tube (Hamamatsu, Shimokanzo, Japan) and a fast sampling oscilloscope of 1 GHz analogue bandwidth (Tektronix 510 5GS/s) was fitted to a monoexponential decay function. From the ring-down time τ_0 in the absence and τ in the presence of analyte the absorbance (in 10-base absorbance units, A.U.) is calculated as

$$\varepsilon C_{eff} l = \left(\frac{\eta_{quartz} \mathrm{L}_{\mathrm{quartz}} + \eta_{air} \mathrm{L}_{\mathrm{air}}}{2.303c} \right) \left(\frac{1}{\tau} - \frac{1}{\tau_0} \right) \qquad \text{(Eq. 1)}$$

where ε is the molar extinction coefficient at 538 nm in $M^{-1}cm^{-1}$, C_{eff} is the effective concentration at the surface layer in M, l is the effective depth of the evanescent wave calculated as 919 nm in this set-up [19], c is the speed of light, η_{quartz} is the refractive index of the prism (1.461), η_{air} is the refractive index of air (1.0008), L_{quartz} is the path length through the prism (9.4 mm) and L_{air} is the path length through the air (59 mm). For non-homogeneous samples the total measured absorbance is the sum of the absorbance by the bulk solution over the effective depth l and the absorbance by surface-adsorbed analytes. The latter will be discussed in more detail in the Results and Discussion section. With the 70-degrees prism, obtainable τ_0 ring-down times were between 25 and 40 ns, and these decreased to 15-20 ns for the highest concentrations of absorber used in this study.

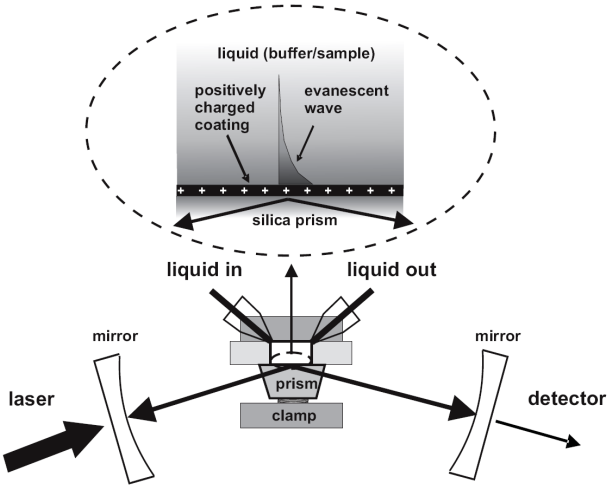

Figure 3.1. Schematic representation of the EW-CRDS set-up, the flow cell attached to the TIR surface, and the evanescent wave probing the sample near the interface.

A 14-µL sized Teflon flow cell was clamped leak-tight on the TIR face of the prism. Cyt *c* adsorption experiments were performed using a stopped-flow approach: 100 µL of a cyt *c* solution was injected in a continuous flow (0.1 mL/min) of 10 mM potassium phosphate buffer (pH 7.4) delivered by a LC pump. When the injection plug completely filled the flow cell, the flow was stopped. The absorbance signal was monitored for a period of 10 to 15 min, after which the flow was restarted and the cyt *c* flushed away with clean buffer solution.

2.3 Coating of the prism

Single-layer PB and triple-layer PB-DS-PB coatings were used in this study. For applying a coating layer on the prism, 100 µL of aqueous polymer solutions was injected and left to react with the surface for 10 min by stopping the flow. For the PB-DS-PB coating, the flow cell was flushed with running buffer for several minutes between application of the polymer layers in order to clean the flow cell of non-bound PB or DS. Irreversibly bound cyt *c* could successfully be removed by injecting 100 µL of 0.1 M sulfuric acid in a flow of 0.05 mL/min. However, in the case of the PB coating this treatment resulted in degradation of the layer, necessitating the complete removal and fresh application of the PB layer. The PB and the PB-DS-PB layers could be removed by injecting, respectively, one or several 500-µL plugs of acetonitrile at a flow of 0.05 mL/min.

2.4. CE system

CE experiments were carried out on a P/ACE MDQ capillary electrophoresis instrument equipped with a UV detector (Beckman Coulter, Fullerton, CA, USA). Fused silica capillaries were obtained from Polymicro Technologies (Phoenix, AZ, USA), having a total length of 30 cm (effective length, 20 cm) and an internal diameter of 50 µm. Hydrodynamic injections were performed using a pressure of 0.5 psi for 4 s. The separation voltage was -30 kV, the capillary temperature was 20 °C and UV absorbance detection was performed at 214 nm. New bare fused-silica capillaries were rinsed with 1 M sodium hydroxide for 30 min at 20 psi, and water for 15 min at 20 psi. After this treatment, the capillaries were coated with the procedure described below. Electropherograms were analyzed using 32 Karat Software, version 7.0 (Beckman Coulter). The electroosmotic mobility (μ_{EOF}) and apparent mobility of cyt *c* (μ_{app}) were calculated as:

$$\mu_{EOF} = \frac{L_{tot} L_{det}}{V t_{EOF}} \qquad \text{(Eq. 2A)}$$

$$\mu_{app} = \frac{L_{tot} L_{det}}{V t_{cyt\,c}} \qquad \text{(Eq. 2B)}$$

where L_{tot} and L_{det} are the total length (30 cm) and length to the detector (20 cm) of the CE capillary, respectively, V is the applied voltage (30 or -30 kV) and t is the migration time (s) of an EOF marker (for μ_{EOF}) or cyt *c* (for μ_{app}). The effective mobility of cyt *c* was subsequently calculated as:

$$\mu_{eff} = \mu_{app} - \mu_{EOF} \qquad \text{(Eq. 3)}$$

The effective mobility (μ_{eff}) may differ from the true electrophoretic mobility due to the contribution of adsorption to the overall mobility.

2.5. Coating of the capillary

Capillaries were coated with a single PB layer by rinsing for 6 min with 10% (w/v) PB solution at 5 psi followed by 3 min with deionized water at 10 psi. For the PB-DS-PB coating, the capillary was successively rinsed 6 min with 10% (w/v) PB solution at 5 psi, 3 min with deionized water at 10 psi, 6 min with 3% (w/v) DS solution at 5 psi, 3 min with deionized water at 10 psi, 6 min with 10% (w/v) PB solution at 5 psi, and 3 min with deionized water at 10 psi. After coating and between runs, capillaries were flushed with fresh BGE for 1 min at 10 psi. Overnight, capillaries were left filled with BGE, with both ends immersed in vials containing BGE.

3. Results and discussion

3.1 EW-CRDS measurements

In a previous publication it was demonstrated that EW-CRDS can be used to monitor cyt *c* adsorption on silica surfaces [16]. Adsorption from protein solutions with concentrations as low as 1 µM could be detected reliably. In the present work, in order to evaluate the effectiveness of coatings for adsorption prevention, surfaces were challenged with much higher protein levels. As a first experiment, the adsorption of cyt *c* to bare silica from a solution of 100 µM in phosphate buffer (pH 7.4) was determined. A typical profile of this EW-CRDS experiment is shown in Figure 3.2A. Immediately after the cyt *c* solution had reached and filled the flow cell, the flow was stopped and the absorbance signal increased up to a practically constant level due to a combination of absorbance by cyt *c* in the bulk solution and by cyt *c* adsorbed to the surface. Starting the flow of neat buffer through the flow cell removed the unadsorbed cyt *c* and potentially desorbs reversibly bound cyt *c*. The absorbance measured 4 min after re-starting the flow was presumed to be caused by irreversibly adsorbed protein. The observed absorbance signal of 7×10^{-4} A.U. (Fig. 2A) agreed well with results on bare silica obtained previously with this system [16]. In addition, as no decrease of the

absorbance signal was observed during the measurement, photodegradation of cyt *c* could be excluded.

The absorbance measured with EW-CRDS could be used to calculate the amount of adsorbed protein on the surface. For the conditions used in our experiments, a maximum cyt *c* saturation surface coverage of 18 pmol/cm^2 has been reported [6,20] which corresponds with 1.1-1.2 pmol present in the laser-illuminated spot of 6-7 mm2. Assuming a protein extinction coefficient of 1.0×10^4 $M^{-1}cm^{-1}$ at 538 nm, the absorbance of a monolayer would be 1.8×10^{-4} A.U. in a conventional transmission geometry at normal incidence. The protein extinction coefficient was determined in house for a cyt *c* solution in the same buffer using a Cary 50 UV-Vis spectrometer. However, a characteristic of evanescent waves is that the absorbance close to the surface is strongly enhanced. For a thin layer with thickness *d* (*d* << penetration depth *l* of the evanescent wave) an enhancement factor [21] comes into play, which was calculated to be 7.4 for the present experimental conditions. Taking this factor into account, the theoretical EW absorbance of a cyt *c* layer was approximately 13×10^{-4} A.U.. So, the observed absorbance signal of 7×10^{-4} A.U. on the bare prism corresponded to approximately one-half of the saturation surface coverage indicating that 0.6 pmol cyt *c* was irreversibly adsorbed at the probed surface spot.

Subsequently, the effect of positively charged coatings on protein adsorption was assessed. A PB single layer and a PB-DS-PB triple layer coating were applied as described in the Experimental section. Using this procedure, a coating layer thickness of the triple layer will be at most 2 nm [22,23], which is very small with respect to the effective penetration depth of 919 nm of the EW-CRDS detection. In other words, the layer will not interfere with the EW-CRDS measurement. Figures 3.2B and C show – on the same absorbance scale as the bare silica measurement – the EW-CRDS profile as obtained for the PB-coated and PB-DS-PB-coated prisms, respectively, after injection of 100 µL of 100 µM cyt *c* in buffer. From the EW-CRDS signal, it was clear that the adsorption of cyt *c* to the prism surface is reduced significantly by coating the fused silica surface with a positively charged layer. After the flow was stopped, the absorbance signal only marginally increased to approximately 0.8×10^{-4} A.U. on both the PB and PB-DS-PB coated prism. Taking the effective penetration depth and cyt *c* extinction coefficient into account, an absorbance signal of 0.9×10^{-4} A.U. could be expected due to bulk absorbance, *i.e.*, in the absence of protein adsorption. When the buffer flow was started again, the measured absorbance returns to baseline level as the cyt *c* leaves the flow cell. So it can be concluded that no measurable amount of cyt *c* was irreversibly adsorbed. Considering the uncertainty (noise) in the absorbance baseline, this implies that with the polymer coatings the protein surface coverage was below 2% when exposed to 100 µM cyt *c*.

A solution of 100 µM cyt *c* was also injected after the prism was coated with a bilayer of PB and DS, which presents a negatively charged surface. After incubation, the buffer flow was started to remove unadsorbed protein from the flow cell. A very high absorbance signal of approximately 4×10^{-3} A.U. remained indicating a strong irreversible adsorption. Actually,

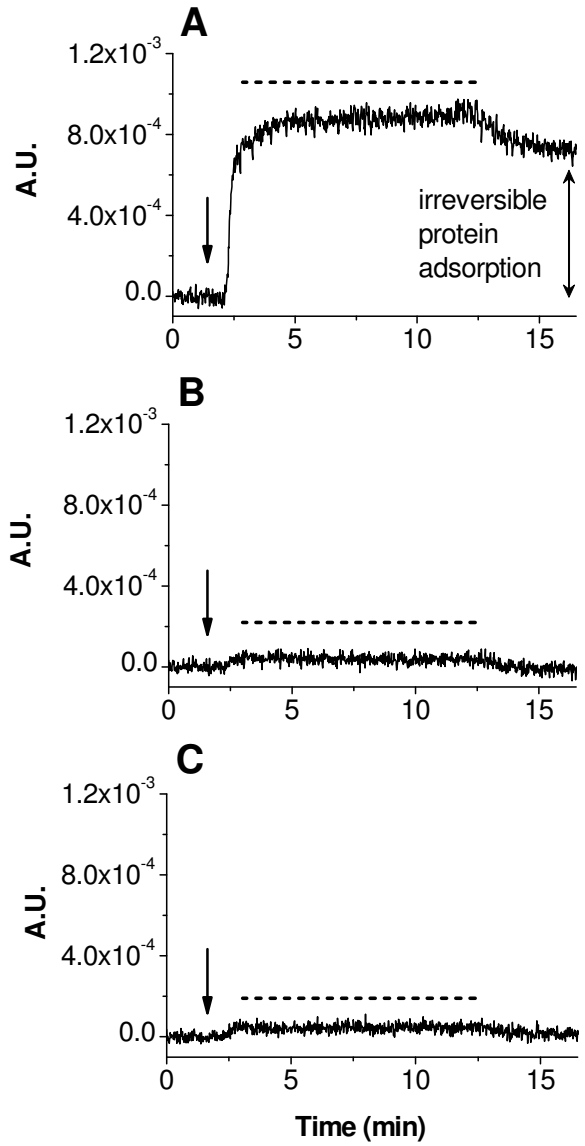

Figure 3.2. EW-CRDS profiles of a (A) bare, (B) PB-coated, and (C) PB-DS-PB-coated silica surface upon exposure to a solution of 100 μM cyt *c*. Arrows indicate the time of cyt *c* injection and the dashed lines indicate the time interval in which the flow was stopped. A.U., absorbance units. Further conditions, see Experimental section.

the EW-CRDS signal indicated that the protein adsorption was 5-6 times higher on the PB-DS bilayer than on bare silica. Apparently, the positively charged protein adsorbed readily to the negatively charged DS. The absorbance signal suggested that the surface was covered with two to three layers of cyt *c*. This value was a rough estimate because the ring-down times became as short as 15 ns and, therefore, could no longer be fitted reliably.

The PB and PB-DS-PB coating were successively exposed to increasing cyt *c* concentrations (100, 200, 400 and 1000 μM) and the increase in surface absorbance (expressed in A.U.) upon each injection was determined by EW-CRDS. Figure 3.3 shows a typical example of the resulting EW-CRDS profile obtained for the triple layer coating. The experiment was performed in triplicate for both coatings and the average amount of adsorbed

protein was calculated (Table 3.1). Protein concentrations up to 200 μM did not result in measurable irreversible protein adsorption. Significant cyt *c* adsorption was observed only for very high protein concentrations (400 and 1000 μM). At a cyt *c* concentration of 400 μM, protein adsorption was similar on the single and triple layer coatings. In this case approximately 2-4% of the surface was covered, which corresponds to 0.02-0.04 pmol of protein adsorbed onto the prism surface. At a cyt *c* concentration of 1000 μM there is a clear difference between the two coatings. On the triple-layer coating a surface coverage of 12% is obtained, corresponding to 0.1 pmol of irreversibly adsorbed protein. The PB single layer yields a surface coverage of 55% (0.6 pmol), which is similar to that observed after injecting 100 μM cyt *c* on bare silica.

When the single- and triple-layer coated silica surfaces were repetitively exposed to 100 and 200 μM cyt *c* over a period of more than two hrs, no measurable irreversible protein adsorption was observed (data not shown). From this result we also conclude that prolonged exposure to protein solutions up to 200 μM does not seriously affect the stability of the PB and PB-DS-PB coating. Irreversibly bound protein – as obtained during exposure to 400 and 1000 μM cyt *c* – was removed by injection of 0.1 M sulfuric acid into the flow cell. This treatment also removed the single PB layer as was clear from the significant protein adsorption observed when the surface was subsequently exposed to 100 μM cyt *c*. Interestingly, the PB-DS-PB coating did not deteriorate upon protein removal with sulfuric acid. The EW-CRDS absorbance signal returned to baseline, whereas the effectiveness against protein adsorption of the triple-layer coating was found to be the same as before the sulfuric acid treatment. As a consequence, the same triple layer could be used during the various

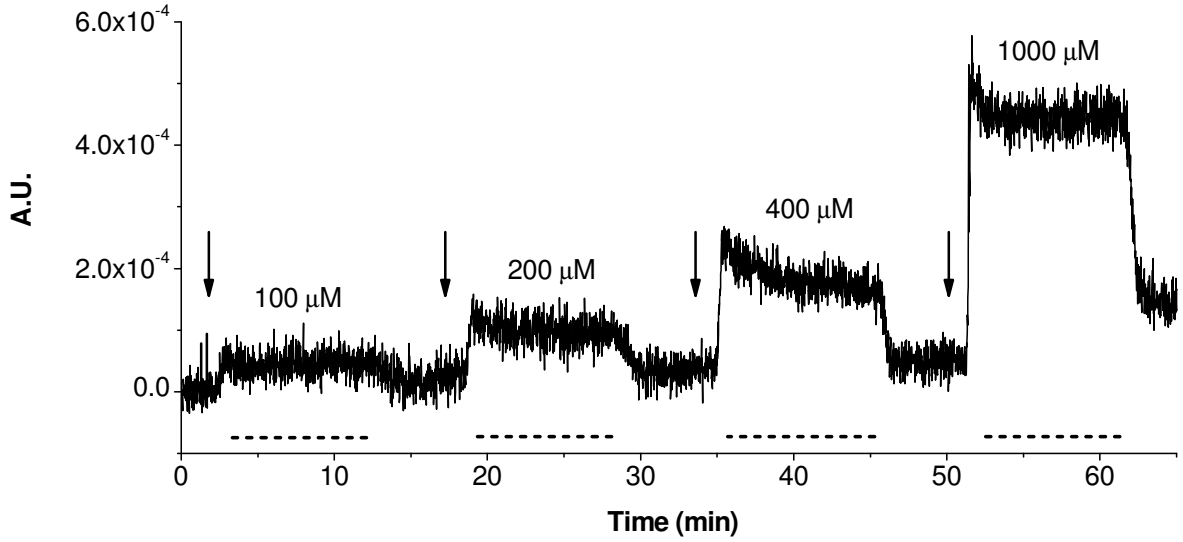

Figure 3.3. EW-CRDS profile of a PB-DS-PB coated silica surface obtained upon successive injections of 100 μL of cyt *c* solutions of increasing concentration. Arrows indicate times of cyt *c* injection and dashed lines indicate time intervals in which the flow was stopped. A.U., absorbance units. Further conditions, see Experimental section.

adsorption experiments without noticeable deterioration. The increased chemical stability of the PB-DS-PB coating with respect to the PB coating was also found by others [17] and may be attributed to stronger interactions between the oppositely charged polymer layers as compared to the silanol-PB interaction [24].

Table 3.1. Irreversible protein adsorption as measured with EW-CRDS on a PB and PB-DS-PB coated prism exposed to different cyt *c* concentrations.

Concentration cyt *c* (μM)	PB coating				PB-DS-PB coating			
	Absorbance[a] (10^{-4} A.U.)	SD[a] (10^{-4} A.U.)	% of saturation coverage[b]	Adsorbed amount (pmol)[b]	Absorbance[a] (10^{-4} A.U.)	SD.[a] (10^{-4} A.U.)	% of saturation coverage[b]	Adsorbed amount (pmol)[b]
100	0.025	0.24	<2.0	<0.02	0.15	0.21	<2.0	<0.02
200	0.15	0.36	<2.0	<0.02	0.33	0.38	<2.0	<0.02
400	0.27	0.17	2.1	0.02	0.44	0.29	3.4	0.04
1000	7.1	0.69	55	0.6	1.5	1.1	12	0.1

a. Average absorbance of 3 measurements; SD, standard deviation.
b. 100% saturation coverage corresponds to a signal of 13×10^{-4} A.U. and 1.15 pmol cyt *c* in the laser-illuminated surface spot.

3.2 CE-UV measurements

To further investigate the performance of the coatings for preventing protein adsorption, CE-UV measurements were carried out. Bare fused-silica (BFS) capillaries were compared to PB and PB-DS-PB coated capillaries. The BGE was 10 mM potassium phosphate (pH 7.4), *i.e.* the same as with the EW-CRDS experiments, whereas solutions of 16 μM cyt *c* were injected. First, a measurement with only formamide (EOF marker) was performed to determine the magnitude of the EOF on the three capillaries (Figure 3.4A, run no. 0). Note that the positively charged coatings resulted in an anodic EOF as opposed to the cathodic EOF that is obtained with BFS.

Ten successive CE analyses of a mixture of EOF marker and cyt *c* were performed on the BFS and both the coated capillaries. On BFS the EOF decreased upon each injection of protein (Figure 3.4A). The decrease can be explained by irreversible protein adsorption of the positively charged protein with the negatively charged capillary wall, which alters the zeta potential at the capillary wall and causes a change of the EOF velocity [4]. Next to the magnitude of the EOF, irreversible protein adsorption was also reflected in the protein recovery. For each cyt *c* peak measured on the coated and non-coated CE capillaries, the recovery was determined relative to the migration time-corrected peak area of cyt *c* as obtained during the first run on the PB-DS-PB coated capillary (Figure 3.4B). On BFS, during the first run no cyt *c* peak could be observed, whereas the other runs resulted in a cyt *c* recovery of less than 10%. In addition, cyt *c* was also repetitively injected on a capillary that was coated with a PB-DS bilayer. No protein peaks were observed whereas the EOF

decreased upon each protein injection (data not shown). Obviously, significant irreversible binding of the protein occurs on both the BFS capillary and the PB-DS coated capillary as was also observed with EW-CRDS.

In contrast to both negatively-charged capillaries, a stable EOF was obtained after application of a positively-charged coating to the silica wall (Figure 3.4A). This indicated that no noticeable irreversible cyt *c* adsorption occurred, most probably due to the electrostatic repulsion between the positively charged coatings and protein molecules. This conclusion is confirmed by the cyt *c* recovery on the coated CE systems which was found to be within 95–110%. The EOF and recovery measurements are in line with the results of the EW-CRDS experiments showing no measurable irreversible protein adsorption at the PB and PB-DS-PB coatings. The magnitude of the EOF is slightly lower with the PB coating than with the PB-DS-PB coating, which might point at incomplete coverage of the silica surface by the coating, resulting in sites for reversible protein adsorption (see below). It should be noted that based on CE results only, it is not possible to fully exclude the possibility of irreversible protein adsorption as recoveries had to be determined relatively (*i.e.*, with respect to the PB-DS-PB coating assuming no protein adsorption). In contrast, EW-CRDS allowed the direct monitoring of (the amount of) adsorbed protein on the silica surface.

Whereas the stability of the EOF and protein recovery reveal the occurrence of irreversible adsorption, the proteins' migration time and peak width can yield information on reversible adsorption [2]. The effective mobility (μ_{eff}) will differ from the true electrophoretic mobility when protein adsorption occurs. As shown in Figure 3.4C, the effective mobility of cyt *c* was approximately 20 times lower on BFS than on the coated capillaries indicating considerable retention and, thus, reversible protein adsorption [25]. A comparison between the PB and the PB-DS-PB coated capillaries reveals a small but significant difference in the effective mobility of cyt *c* (Figure 3.4C). Because cyt *c* migrated slower than the EOF when using positively charged coatings, the somewhat higher mobility obtained with the single PB layer indicates retention and, therefore, reversible adsorption.

Determination of the plate number of the cyt *c* peak over 10 runs on the three systems (Figure 3.4D) showed that the lowest plate numbers are obtained on BFS. This is evidently caused by the significant reversible adsorption of cyt *c* to the capillary wall which leads to extra band broadening due to the resistance to mass transfer [26]. Indeed, cyt *c* plate numbers for the coated capillaries were higher than for BFS. Still, the cyt *c* plate number for the PB single layer was lower than for the PB-DS-PB coating and a decrease each run was observed. This might be due to the partial detachment of PB during the successive runs, although it should be noted that the recovery (and thus irreversible adsorption) did not significantly change during the successive injections of cyt *c*. The PB-DS-PB triple layer coating clearly provides the highest and most stable plate numbers. This was also previously found by us [18] and others [17], and could be contributed to the more effective suppression of reversible protein adsorption by the triple-layer coating.

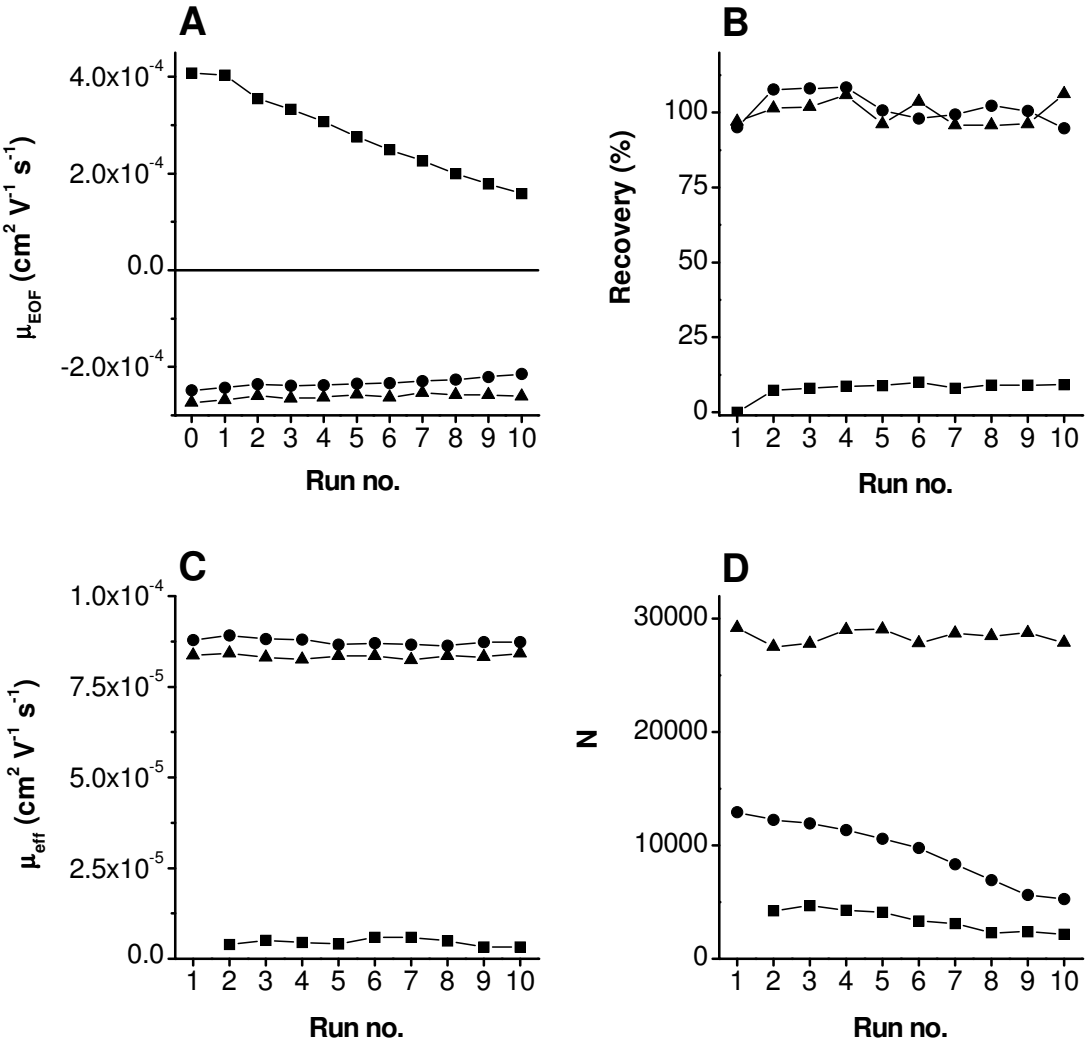

Figure 3.4. (A) EOF mobility, and (B) recovery, (C) effective mobility and (D) plate number of cyt *c* obtained during CE-UV analyses of 0.2 v/v% formamide (run no. 0) or a mixture of 0.2 vol% formamide and 16 µM cyt *c* (runs no. 1-10) using a (■) BFS capillary, and a (●) PB and (▲) PB-DS-PB coated capillary. With BFS, no cyt *c* was detected during run no. 1. Voltage, 30 kV (BFS) and -30 kV (coated capillaries). Further conditions, see Experimental section.

4. Conclusions

The effectiveness of charged noncovalent polymer capillary coatings to prevent adverse adsorption of basic proteins to a silica surface was studied by EW-CRDS and CE-UV. EW-CRDS is particularly useful for direct probing of irreversible protein adsorption. The EW-CRDS results showed that for cyt *c* concentrations up to 200 µM, PB and PB-DS-PB coatings effectively suppress irreversible adsorption. The triple layer coating appeared to be most stable when exposed to high protein concentrations and sulfuric acid. CE-UV results on EOF and cyt *c* peak area confirmed that the PB and PB-DS-PB coatings strongly reduce irreversible adsorption of basic proteins. It should be noted that – in contrast to EW-CRDS – CE-UV provides a relative measure for irreversible adsorption. On the other hand, monitoring

of electrophoretic protein mobilities and plate numbers as determined with CE-UV allows the evaluation of reversible protein adsorption which is not easy to assess with EW-CRDS. It follows that especially the BFS capillary, but also the PB-coated capillary, exhibits reversible adsorption, whereas this phenomenon is most effectively minimized by the PB-DS-PB coating. This led to the most optimum plate numbers for cyt *c* on the triple layer coating. Overall, from this study it can be concluded that EW-CRDS and CE-UV provide complementary information about the nature and magnitude of basic protein adsorption, and show good potential for the evaluation of silica coating materials.

Further improvement of the EW-CRDS set up would allow detection of lower amounts of adsorbed protein. Enhancement of the sensitivity could, for example, be achieved by employing the Soret band instead of the Q band of cyt *c* by using a laser in the blue region of the spectrum in combination with the appropriate mirrors. At the moment we are studying this option. For other types of proteins that do not have a strong absorption in the visible range, there are also options to carry out CRDS measurements in the UV range, although at very short wavelengths currently the mirror quality often still is the limiting factor [27]. We also will use EW-CRDS and CE-UV for the study of the adsorption of acidic proteins on bare and coated silica surfaces.

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Chapter 4

Capillary electrophoresis of intact basic proteins using noncovalently triple-layer coated capillaries

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Abstract

The usefulness of a noncovalent, positively-charged capillary coating for the efficient analysis of intact basic proteins with capillary electrophoresis (CE) was studied. Capillaries were coated by subsequent flushing with solutions of 10% (w/v) Polybrene (PB), 3% (w/v) dextran sulfate (DS) and again 10% (w/v) PB. Coating characterization studies showed that stable coatings could be produced which exhibited a pH-independent and highly reproducible electroosmotic flow (EOF). The PB-DS-PB coating was evaluated with TRIS phosphate background electrolytes (BGEs) of various pH using the four basic model proteins α-chymotrypsinogen A, ribonuclease A, cytochrome *c* and lysozyme. Typical migration time RSDs for the proteins were less than 0.85%, and plate numbers were above 125,000. The high separation efficiency allowed detection of several minor impurities in the model proteins. Using a BGE of medium pH, the CE system with triple-layer coating appeared to be useful for the repeatable profiling of recombinant humanized monoclonal immunoglobulin G_1 showing a characteristic pattern of glycoforms. The CE system was also applied to the characterization of two llama antibodies, which were produced in *Saccharomyces cerevisiae*, revealing the presence of a side product in one of the antibodies. The high migration time stability allowed the reliable determination of antibody-antigen binding by monitoring migration time shifts. Finally, the feasibility of using the PB-DS-PB coated capillaries for CE with mass spectrometric detection was shown by the characterization of the impure llama antibody sample.

1. Introduction

Biopharmaceuticals are becoming more and more important in drug development, and an increasing number of registered drugs are proteins. A survey from 2006 showed that at the time over one hundred pharmaceutical proteins had been approved for marketing [1]. Today, several hundreds of protein drugs are in clinical trials for treatment of HIV, cancer and a number of other illnesses, including autoimmune, genetic, growth, neurological and skin disorders. Due to these advancements, one can observe a growing demand for separation methodologies that allow characterization of intact proteins. Separation techniques frequently used for protein analysis are slab-gel electrophoresis (SGE), liquid chromatography (LC) and capillary electrophoresis (CE). SGE is an established technique for protein separation. Although many samples can be analyzed simultaneously and a relatively large number of proteins can be resolved applying two-dimensional SGE, it also has limitations. Analyses are relatively long and labor-intensive, detection can not be done on-line, and it lacks precise (and automated) quantitation. LC is advantageous due to its separation power, ease of automation and routine coupling with various detection principles, like mass spectrometry (MS). However, adverse interactions of proteins with the stationary phase and denaturation of the proteins in organic mobile phases can seriously decrease separation efficiency and resolution. Capillary electrophoresis (CE) is a separation technique that shows attractive features for the analysis of proteins. In general, CE provides high plate numbers (narrow peaks), analysis times are relatively short, and the amount of sample needed is minute. Furthermore, analysis of proteins can be carried out under mild conditions without the need for organic solvents or very high salt concentrations. Finally, free zone electrophoresis migration is a function of charge, size and shape of a compound, representing a unique separation mechanism. In combination with the high efficiency, relatively small differences among proteins may be sufficient for their separation.

One of the potential problems of CE of proteins is that the separation can be hampered by adsorption of the proteins onto the fused-silica capillary wall. Adsorption in principle can be (partly) avoided using background electrolytes (BGEs) of extreme pH and high ionic strength. However, the applicability of these approaches may be limited due to protein stability problems. Therefore, the more common approach in protein CE is to coat the capillary wall with a non-adsorptive agent. Over time, varying types of capillary coatings have been proposed [2-10]. Covalently attached coatings can be very effective, however, their production can be tedious and time-consuming and may not be reproducible. Noncovalently attached coatings offer a more practical solution, as they can be made by simply flushing the capillary with the solution of the proper coating agent. These coatings are based on the physical adsorption caused by electrostatic interaction between the coating agent and the capillary wall. Therefore, the coating procedure is relatively fast and can be easily repeated.

Recently, we have shown the usefulness of capillaries that were noncovalently coated with a bilayer of Polybrene (PB) and poly(vinylsulfonic acid) (PVS) for the fast, reproducible

and highly efficient separation of acidic proteins. The applicability of the bilayer coating for the analysis of acidic protein drugs was demonstrated [11-13]. However, this type of coating is not suitable for the analysis of basic proteins as the outer coating layer is negatively charged and will induce adsorption of positively charged proteins. In order to circumvent adverse analyte-wall interactions for basic proteins (pI > 8), coatings exhibiting a positively charged outer layer would be more suitable. A straightforward solution would be to use a single layer of PB as positively-charged coating. However, in our and others' [14] hands such a coating lacks prolonged stability and would need run-to-run regeneration to assure constant performance. Various other positively charged coating agents have been described in literature [10,14-21]. An interesting and highly flexible approach, introduced by Katayama *et al*. [14], is the use of successive multiple ionic polymer layers to construct stable coatings. Katayama and coworkers proposed a triple-layer coating of successive layers of PB, dextran sulfate (DS) and PB for the efficient CE analysis of basic proteins. First results were very promising, however, emphasis in their study was only on the analysis of basic proteins at low pH. The applicability of the CE system to relevant protein samples was not demonstrated and the potential of the PB-DS-PB coating for CE-MS of proteins was not investigated.

In this work the stability, reproducibility and CE performance for basic proteins of PB-DS-PB coated capillaries is extensively studied. Particular attention is paid to the possibility to use the PB-DS-PB coating at various pH values. Moreover, the applicability of the CE system is studied by the profiling of immunoglobulin G_1 at medium pH, and the analysis of basic llama antibodies (including their binding products) at low pH. The feasibility of using the triple-layer coating in conjunction with MS detection for protein analysis is investigated by the characterization of an impure llama antibody sample.

2. Materials and methods

2.1. Chemicals

Polybrene (hexadimethrine bromide, PB) and dextran sulfate (DS) sodium salt were purchased from Sigma-Aldrich (Steinheim, Germany). The BGE constituents acetic acid (99.9%), sodium hydroxide, hydrochloric acid (37%), TRIS base, phosphoric acid (85%), sodium dihydrogen phosphate and disodium hydrogen phosphate were obtained from Merck (Darmstadt, Germany). For EOF determinations, formamide from Fluka (Steinheim, Germany) was used in a final concentration of 0.1% (v/v) in deionized water.

Stock solutions (1 mg/mL) of the proteins α-chymotrypsinogen A, ribonuclease A, cytochrome *c* and lysozyme (all from Sigma-Aldrich) were prepared in deionized water. A protein test mixture with 250 µg/mL of each protein was made by mixing equal volumes of each protein stock solution. A stock solution of 1.0 mg/mL of recombinant humanized monoclonal immunoglobulin G_1 (IgG_1; molecular weight, 149 kDa) was in 100 mM sodium phosphate (pH 7.2). For CE analysis, the IgG_1 solution was diluted in deionized water to a

final concentration of 125 µg/mL. Llama antibodies VHH-A52 against the azo-dye RR1 and VHH-R2 against the azo-dye RR6 were produced in *Saccharomyces cerevisiae* via batch fermentation [22]. Llama antibody stock concentrations of 161 µM VHH-A52 and 70 µM VHH-R2 were in phosphate buffered saline (145 mM NaCl, 7.5 mM Na_2HPO_4, 2.5 mM NaH_2PO_4, 0.01% (w/v) NaN_3). VHH-A52 was diluted to 70 µM with 10 mM TRIS phosphate (pH 7.0). The azo-dye RR1 (Cibacron Red) was obtained from Ciba Geigy (Basel, Switzerland) and RR6 (Procion Rubine) from ICI (Runcorn, UK). Stock solutions (100 mM) of the azo-dyes were prepared in 10 mM ammonium chloride and were diluted to 7 mM with 10 mM TRIS phosphate (pH 7.0). Binding experiments were performed by mixing 5 µL azo-dye solution (7 mM) with 5 µL VHH solution (70 µM). The mixture was led to react for 15 min at room temperature. All VHH samples were diluted to a final VHH concentration of 14 µM with 10 mM TRIS phosphate (pH 7.0) prior to CE analysis.

BGEs of 55 mM (pH 3.0) and 38 mM (pH 6.0) phosphate were prepared by respectively dissolving 0.0927 mL phosphoric acid and 0.135 g sodium dihydrogen phosphate in 25 mL deionized water. BGEs of 285 mM (pH 4.0) and 73 mM (pH 5.0) acetate were prepared by respectively diluting 0.4 mL and 0.103 mL acetic acid to 25 mL in deionized water. BGEs of 54 mM (pH 7.0) and 85 mM (pH 8.0) TRIS were prepared by dissolving 0.164 g and 0.256 g of TRIS base in 25 mL in deionized water, respectively. The pH of these six BGEs was set to the desired value with either HCl or NaOH, while the ionic strength was set to 0.05 M with NaCl. BGEs of TRIS phosphate were prepared by weighing TRIS to the desired concentration followed by titration to the appropriate pH with phosphoric acid. A BGE of 50 mM sodium phosphate (pH 6.8) was prepared by mixing equimolar solutions of sodium dihydrogen phosphate and disodium hydrogen phosphate in the appropriate ratio to reach pH 6.8. A BGE of 175 mM acetate (pH 2.7) in deionized water was prepared by diluting 100 µL acetic to 10 mL with deionized water.

2.2. CE system

CE experiments were carried out on a P/ACE MDQ capillary electrophoresis instrument equipped with a UV detector (Beckman Coulter, Fullerton, CA, USA). Fused silica capillaries were from Polymicro Technologies (Phoenix, AZ, USA), having a total length of 40 or 60 cm (effective length, 30 or 50 cm, respectively) for CE-UV and 100 cm for CE-MS. The capillaries had an internal diameter of 50 µm. Hydrodynamic injections were performed using a pressure of 0.5 psi for 6 sec or 13 sec (40 cm and 60 cm capillary, respectively) or 1.5 psi for 13 sec (100 cm capillary). Unless stated otherwise, the separation voltage was -30 kV, the capillary temperature 20 °C and detection was performed at 214 nm. Electropherograms were analyzed using 32 Karat Software, version 7.0 (Beckman Coulter). New bare fused-silica capillaries were rinsed with 1 M NaOH for 30 min at 20 psi, and water for 15 min at 20 psi. After this treatment, capillaries were coated with the procedure described below.

2.3. Capillary coating

For the PB-DS-PB coating, solutions of 10% (w/v) PB and 3% (w/v) DS in deionized water were prepared. The solutions were filtered over a 0.45 µm filter type HA (Millipore, Molsheim, France) prior to use. Capillaries were coated by subsequently rinsing with 10 capillary volumes of 10% (w/v) PB solution at 5 psi, 10 capillary volumes of deionized water at 10 psi, 10 capillary volumes of a 3% (w/v) DS solution at 5 psi, 10 capillary volumes of deionized water at 10 psi, 10 capillary volumes of a 10% (w/v) PB solution at 5 psi, and 10 capillary volumes of deionized water at 10 psi. The capillary was then ready for CE analysis with the BGE of choice. Between runs, coated capillaries were flushed with fresh BGE for at least 3 capillary volumes at 10 psi. During the analysis of the monoclonal antibody, an additional flush of 3 capillary volumes at 10 psi with 20 mM phosphoric acid (pH 4.0) was performed between runs. Overnight, capillaries were filled with BGE and tips were immersed in vials with BGE.

2.4. Mass spectrometry

MS was performed using a micrOTOF orthogonal-accelerated TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) using electrospray ionization (ESI) in the positive ion mode. Transfer parameters were optimized for the mass range 500-3000 *m/z* by direct infusion of an ESI tuning mix (Agilent Technologies, Waldbronn, Germany). CE-MS coupling was realized by a co-axial sheath-liquid interface (Agilent Technologies) with isopropanol-water-acetic acid (50:50:0.1, v/v/v) as sheath liquid. The sheath liquid was delivered by a 2.5 mL gas-tight syringe (Hamilton, Reno, NV, USA) using a syringe pump of Cole-Parmer (Vernon Hill, IL, USA). The following interface conditions were used: sheath-liquid flow, 4 µL/min; dry gas temperature, 180 °C; nitrogen flow, 4 L/min; nebulizer pressure, 0.4 bar. The ESI voltage was -5.0 kV. CE-MS data were analysed using Bruker Daltonics Data Analysis software.

3. Results and discussion

3.1. Coating characterization

We first considered the use of a single layer of PB as potential coating, and studied its endurance. PB was applied to the capillary and, subsequently, thirty repetitive analyses of formamide were performed using a TRIS phosphate BGE of pH 8.0. During each measurement the voltage was applied for 30 min and in between measurements the capillary was flushed with fresh BGE. Figure 4.1 depicts the resulting magnitude of the EOF. It appears that the single PB layer initially does not create a constant EOF. From run 7 till 20 the EOF is stable, whereas it rapidly drops after 20 runs. After run 25, the magnitude of the EOF could no longer be determined. Probably, the direction of the EOF became anodic as the PB detached of the capillary wall. As firstly demonstrated by Katayama *et al*. [14], noncovalent

coatings are more stable when applying multiple layers. Indeed, when producing a bilayer of PB-DS, the EOF – which now is towards the anode – was much more stable (Figure 4.1). When applying another layer of PB, the resulting positively charged coating shows a prolonged stability with hardly any change in EOF over 30 runs (RSD is 2.4%), which equals 900 min of analysis time.

Subsequently, the EOF was determined in triplicate in the pH range from 3.0 to 8.0 using 100 mM TRIS phosphate BGEs. At all pH values the repeatability of the magnitude of the EOF was less than 0.6% (n=3). It was observed that with increasing pH, the EOF decreases from $-3.38 \cdot 10^{-8}$ m^2 V^{-1} s^{-1} (pH 3.0) to $-1.33 \cdot 10^{-8}$ m^2 V^{-1} s^{-1} (pH 8.0). However, this change should not be attributed to the difference in pH, but to the change in ionic strength of the different BGEs. To observe whether the magnitude of the EOF was truly pH-independent, BGEs in the pH range from 3.0 to 8.0 with an equal ionic strength (0.05 M) were prepared. With these BGEs the EOF was subsequently determined in triplicate. Now, the EOF varied between $-2.79 \cdot 10^{-8}$ m^2 V^{-1} s^{-1} (pH 3.0) and $-2.54 \cdot 10^{-8}$ m^2 V^{-1} s^{-1} (pH 7.0) and showed to be pH-independent. In addition, the repeatability of the EOF magnitude with these BGEs of equal ionic strength is again excellent, with RSD values less than 0.8% (n=3).

In order to check the reproducibility of the production of the triple-layer coating, ten capillaries were prepared with the PB-DS-PB coating. Subsequently, the EOF was determined on five capillaries in triplicate using a TRIS phosphate BGE of pH 8.0 and also on five capillaries in triplicate using a BGE of acetic acid (pH 2.7). The results in Table 4.1 show that the PB-DS-PB coating can be made in a very reproducible way providing very similar EOFs among different capillaries. The difference in the EOF magnitude at low and medium pH can be explained by differences in ionic strength amongst the BGEs. With both BGEs good capillary-to-capillary reproducibility (RSD < 0.9%) for the EOF magnitude are obtained.

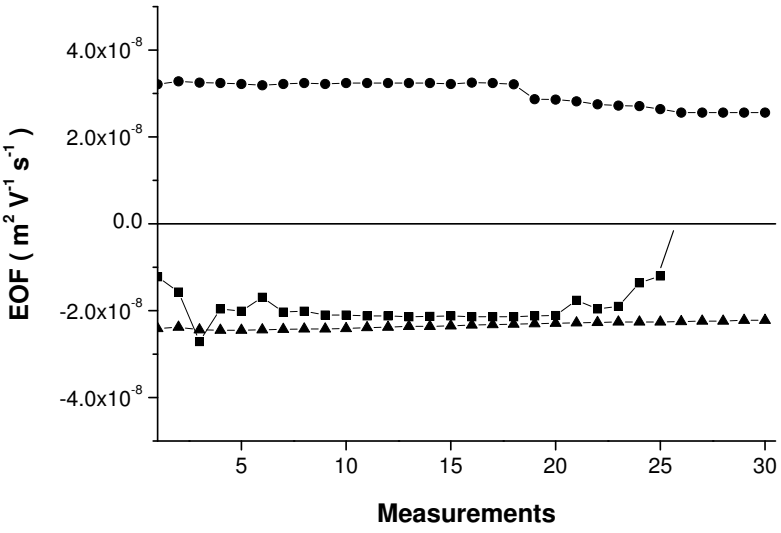

Figure 4.1. Magnitude of the EOF of (■) PB mono-layer, (●) PB-DS bilayer and (▲) PB-DS-PB triple-layer coated capillaries during 30 measurements of 30 min. Conditions: capillary length, 40 cm; BGE, 50 mM TRIS phosphate (pH 8.0); voltage, 30 kV (bilayer system) and -30 kV (mono- and triple-layer system).

Table 4.1. Run-to-run repeatability (n=3) and capillary-to-capillary reproducibility (n=5) of the EOF using BGEs of medium and low pH. Conditions: capillary length, 40 cm; capillary coating, PB-DS-PB.

	Run-to-run		Capillary-to-capillary	
BGE	EOF ($m^2 V^{-1} s^{-1}$)	RSD (%)	EOF ($m^2 V^{-1} s^{-1}$)	RSD (%)
100 mM TRIS phosphate (pH 8.0)	$-1.34 \cdot 10^{-8}$	0.20	$-1.33 \cdot 10^{-8}$	0.89
175 mM acetic acid (pH 2.7)	$-5.79 \cdot 10^{-8}$	0.18	$-5.81 \cdot 10^{-8}$	0.63

3.2. CE of basic model proteins

In order to verify the utility of the triple-layer coating for the separation of basic proteins, a mixture of α-chymotrypsinogen A (p*I* 9.0), ribonuclease A (p*I* 9.7), cytochrome *c* (p*I* 10.5) and lysozyme (p*I* 11.0) was analyzed in triplicate using 100 mM TRIS phosphate BGEs of different pH. The PB-DS-PB coating appeared to be stable under all conditions providing repeatable results. With all BGEs migration time RSDs for the four proteins of less than 0.85% (n=3) were obtained (Table 4.2). Apparently, protein adsorption is effectively prevented so that narrow and quite symmetrical peaks are obtained. Typical plate numbers that were obtained over the pH-range from 3.0 to 8.0 are between 125,000 and 200,000 without BGE optimization.

Figure 4.2 shows the electropherograms of the test mixture analyses with the BGEs of different pH. As the basic proteins are positively charged and a reverse polarity is applied, they migrate after the EOF marker. Between pH 5.0 and 8.0 the proteins appear to migrate in the order of their isoelectric point (α-chymotrypsinogen A first and lysozyme last) and their effective mobilities do not change significantly. As the EOF magnitude also does not change severely, migration times remain similar. At low pH the effective mobilities increase rapidly, which is most probably due to an increase of the proteins' net positive charge as result of protonation of the carboxylic acid groups in the protein chains. However, as the EOF magnitude also increases rapidly at low pH, migration times are shorter than at medium pH. Furthermore, at low pH the migration order of cytochrome *c* and lysozyme changes. This can be explained by the stronger increase of the net positive charge at low pH in cytochrome *c* compared to lysozyme as was calculated using Protein Calculator v3.3 [23]. Overall, these results show that the triple-layer coating can be applied over a broad pH-range for protein analysis. However, optimal separation conditions vary per protein and should be optimized for the protein of interest.

Interestingly, the high efficiency revealed the presence of a series of small peaks in the electropherograms of the protein mixture (Figure 4.2B). These peaks originate from impurities in the test proteins and are most likely protein degradation products. By injection of separate protein standards, it could be determined that the impurities have a shorter migration time, and thus are less positively charged or larger, than the protein they originate

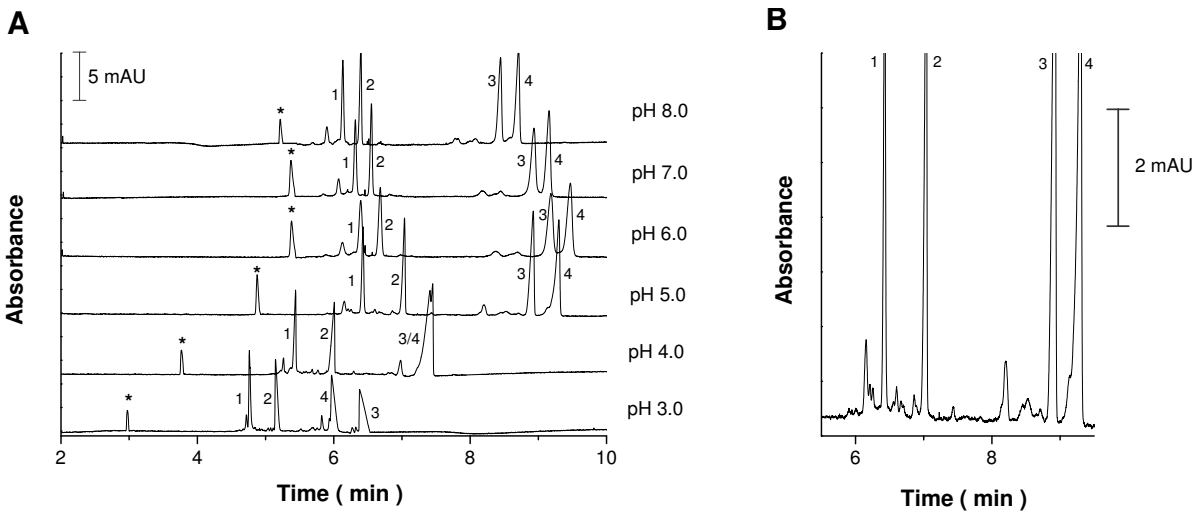

Figure 4.2. (A) CE-UV analyses of a mixture of formamide (0.01 vol%), α-chymotrypsinogen A (1), ribonuclease A (2), cytochrome *c* (3) and lysozyme (4) (250 µg/mL each) using 100 mM TRIS phosphate BGEs of different pH. Formamide (*) is used as EOF marker. (B) Detail of the electropherogram obtained with a BGE of 100 mM TRIS phosphate (pH 5.0). Conditions: capillary coating, PB-DS-PB; capillary length, 40 cm.

Table 4.2. Migration time RSDs (%; n=3) for intact basic proteins using 100 mM TRIS phosphate BGEs of different pH. Conditions: capillary length, 40 cm; capillary coating, PB-DS-PB.

pH	α-Chymotrypsinogen A	Ribonuclease A	Lysozyme	Cytochrome *c*
3.0	0.39	0.44	0.68	0.81
4.0	0.34	0.37	0.44	0.47
5.0	0.49	0.55	0.64	0.66
6.0	0.64	0.47	0.83	0.74
7.0	0.24	0.12	0.51	0.31
8.0	0.22	0.63	0.20	0.78

from. To unambiguously assign these impurities, other methodologies, like *e.g.* mass spectrometry, have to be applied.

3.3. CE of basic antibodies

3.3.1. Monoclonal IgG_1 antibody

Over the last years, monoclonal antibodies have been introduced for the treatment of several diseases, such as HIV, autoimmune disorders and cancer [24]. Monoclonal antibodies are subject to post-translational modifications that occur during the production of the protein. As monoclonal antibodies are large proteins, with an approximate weight of 150 kDa, it is interesting to investigate whether the PB-DS-PB coated capillaries would be suitable for their analysis and allow profiling of possible isoforms. A recombinant humanized monoclonal IgG_1, with a p*I* of approximately 9.0, was used as test compound.

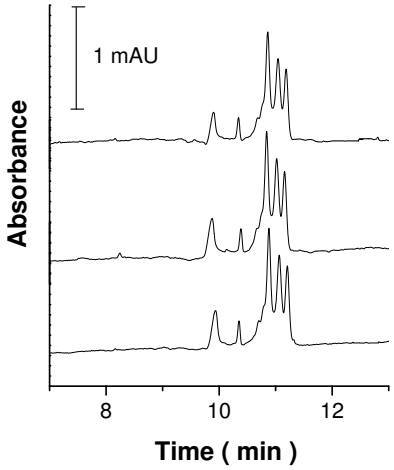

Figure 4.3. Repeated CE-UV analyses (n=3) of 125 µg/mL recombinant humanized monoclonal IgG_1. Conditions: capillary coating, PB-DS-PB; capillary length, 60 cm; BGE, 50 mM phosphate (pH 6.8); capillary temperature, 15°C.

The antibody was analyzed using a 50 mM phosphate BGE (pH 6.8) on a 60 cm capillary. Under these conditions, IgG_1 shows a repeatable pattern of partially resolved bands in the 10.5-11.5 min region. The migration time RSDs of the main three peaks were less than 0.55% (n=3) (Figure 4.3). The observed profile of peaks most probably is caused by different glycoforms. IgG_1 contains two possible glycosylation sites [25] that are susceptible to a broad range of glycan modifications [26]. Amongst them are different amounts of sialic acid groups and thus negative charges. The identity of the peaks migrating at 9.9 and 10.3 min is unknown, but they most probably are not proteinaceous. Isoforms are not expected to have such deviating migration times, whereas the sample is known not to contain heavy or light chains resulting from IgG reduction [27]. The combination of CE, employing PB-DS-PB coated capillaries, with mass spectrometry could in principle be used for the assignment of the isoforms and other sample components. We are currently studying this option.

3.3.2. Llama antibodies

In the mid-1990s it was reported that camelids contain a unique class of antibodies that lack light chains [28] and offer several advantages over normal antibodies. These include, amongst others, a high chemical stability and nanomolar affinity [29-31]. The binding part of these so-called heavy-chain antibodies comprises only a 15 kDa single domain, called VHH. During antibody engineering, the VHHs are purified using, for example, ultrafiltration [31] or protein A columns [32]. CE could be an effective tool for the characterization of the purified products. We studied the potential of PB-DS-PB coated capillaries for the purity analysis of single domain binding fragments VHH-A52 (p*I* 7.9) and VHH-R2 (p*I* 9.2) derived from llamas. The antibodies were respectively raised against azo-dye RR1 and azo-dye RR6.

After evaluating several BGE constituents at various pH-values and concentrations, it was observed that the best peak shapes and separation efficiency were obtained at low pH. A

BGE of 175 mM acetic acid (pH 2.7) resulted in the best separation efficiency and was used for further research. With this volatile BGE narrow, symmetrical peaks (Figure 4.4) and migration time RSD-values of less than 0.45% were obtained. Clearly, the CE system is suitable for llama antibody analysis. Analysis times are short due to the high velocity of the EOF generated by the coated capillary for a low ionic strength BGE. VHH-R2 migrates at 1.85 min and shows no impurities, whereas for theVHH-A52 sample two major components are completely resolved in less than 1.8 min. In addition, a small peak migrating at 1.95 min is also observed in the VHH-A52 sample

Next, it was investigated whether antigen binding to the llama antibody can be probed with the CE system. Azo-dye RR1 was added in a 100 molar excess to VHH-A52, whereas RR6 was added in the same excess to VHH-R2. After 15 min incubation at room temperature, the two samples were injected. VHH-R2 mixed with azo-dye RR6 shows a single peak with a migration time shorter than that of pure VHH-R2 (Figure 4.4D). The analysis of the mixture containing VHH-A52 and azo-dye RR1 shows two components with shorter migration times, but a peak pattern similar to VHH-A52 alone (Figure 4.4C). Furthermore, in the latter electropherogram, a third small peak is observed at approximately the same migration time (1.95 min) as prior to azo-dye addition. The negatively charged azo-dyes are not detected due to their lack of absorbance at 214 nm. The difference in migration times of the dye-antibody complex and the antibody can be explained by multiple negative charges (*i.e.* sulfonate groups) in the azo-dyes. Therefore, the complexes migrate faster than the respective VHHs. The resemblance in peak patterns resulting from the VHH-A52/azo-dye sample suggests that

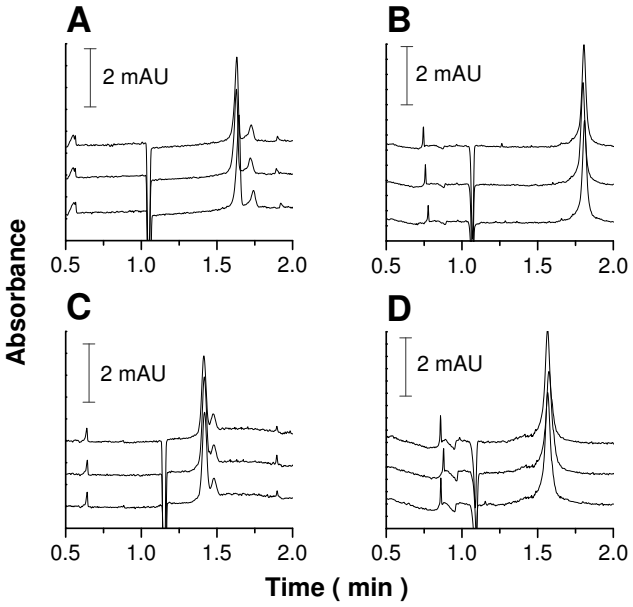

Figure 4.4. Repeated CE-UV analyses (n=3) of (A) 14 µM VHH-A52, (B) 14 µM VHH-R2, (C) 70 µM VHH-A52 incubated with 7 mM RR1 for 15 min and (D) 70 µM VHH-R2 A52 incubated with 7 mM RR6 for 15 min. The samples in (C) and (D) were diluted to a VHH concentration of 14 µM prior to CE analysis. Conditions: capillary coating, PB-DS-PB; capillary length, 40 cm; BGE, 175 mM acetic acid (pH 2.7).

the two components both are llama antibodies showing antigen binding activity. The small peak at 1.95 min (Figure 4.4C) most probably is not a VHH-A52 modification, or lacks binding activity, as no shift in migration time is observed. Considering the small migration time RSDs for the VHHs, it can be reliably concluded that the migration time shift results from dye-complex formation and are not caused by system instability. Interestingly, as the peaks of the VHH-azo-dye complexes are symmetrical and narrow, the low pH did not appear to induce dissociation of the VHH/azo-dye complexes.

3.4. CE-MS using PB-DS-PB coating

The combination of CE and electrospray ionization-mass spectrometry (ESI-MS) provides a powerful tool for the separation and characterization of intact proteins and their potential impurities [33]. Therefore, we studied the feasibility of using triple-layer coated capillaries for the CE-MS analysis of proteins. As a test sample, the impure llama antibody VHH-A52 was analyzed. As with CE-UV, the volatile BGE of 175 mM acetic acid (pH 2.7) was applied in combination with a capillary that was coated with PB-DS-PB. Subsequently the capillary was positioned in the co-axial sheath-liquid sprayer and the interface was placed in the ESI source of a time-of-flight (TOF) mass spectrometer. Figure 4.5A depicts the CE-MS result obtained for the VHH-A52 sample showing a peak pattern similar to the one found with CE-UV (Figure 4.4A). Importantly, no signals of PB were observed in the mass spectra during the complete run, indicating that the coating is stable and does not detach from the capillary wall, and thus will not interfere with MS detection.

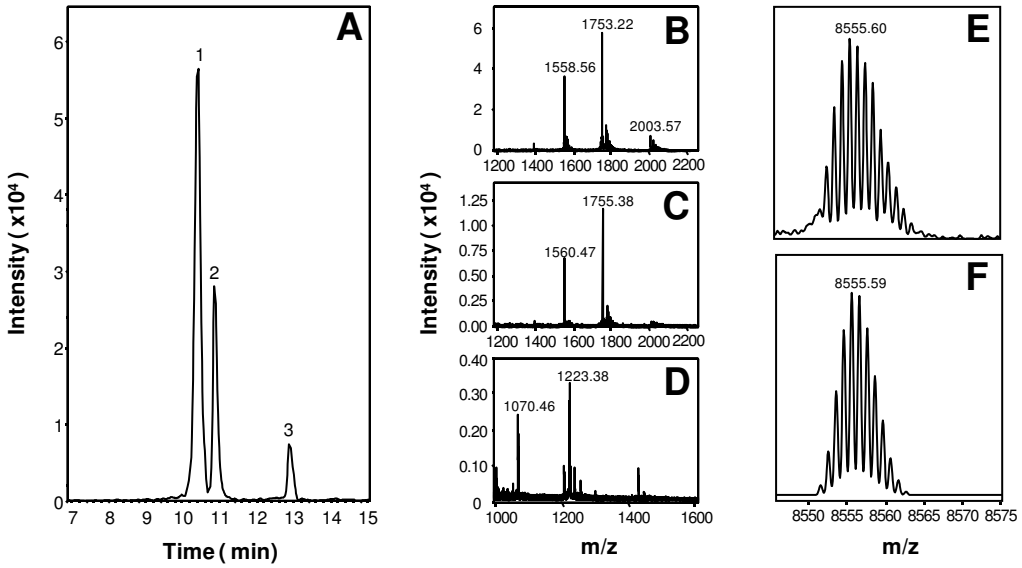

Figure 4.5. CE-ESI-TOF-MS of 14 µM VHH-A52. (A) Base-peak electropherogram constructed in the mass range *m/z* 1000-3000. (B-D) Average mass spectra obtained from peak 1, 2 and 3, respectively. (E) Deconvoluted mass spectrum of peak 3. (F) Theoretically calculated mass spectrum of *S. cerevisiae* ubiquitin ($C_{375}H_{625}N_{105}O_{120}S_1$). Conditions: capillary coating, PB-DS-PB; capillary length, 100 cm; BGE, 175 mM acetic acid (pH 2.7).

The efficient CE separation in combination with the high mass accuracy data obtained with TOF-MS now allows characterization of the sample constituents. Deconvolution of the recorded mass spectra (Figures 4.5B-D) of the separated compounds yield masses of 14017.9 (peak 1), 14034.9 (peak 2) and 8555.6 Da (peak 3), respectively. Based on the mass difference, we presume that peak 1 represents a modification of the VHH-A52 form represented by peak 2, involving the transition of N-terminal glutamine to pyroglutamic acid (http://unimod.org/modifications_list.php). This results in a mass difference of 17 Da and the loss of positive charge. The latter is reflected in the shorter migration time of the modified antibody (peak 1) with respect to the unmodified antibody (peak 2). Considering the results described in Section 3.3.2, the modification apparently does not affect the antigen binding capacity of the antibody. Based on its mass spectrum (Figure 4.5E) peak 3 could be identified as the protein ubiquitin from *S. cerevisiae* [34], as also confirmed by comparison with the theoretically calculated mass spectrum of this protein (Figure 4.5F). As was concluded from the CE-UV experiments described above, ubiquitin is not expected to show antigen binding activity.

4. Concluding remarks

The usefulness of PB-DS-PB coated capillaries for CE–UV analysis of intact basic proteins using BGEs in the pH range from 3.0 to 8.0 was shown. The preparation of the coating is simple and fast comprising flushing of the capillary with solutions of the coating agents. The coating proved to be stable, exhibiting a pH-independent and highly reproducible EOF. The coating can be used in combination with a broad range of BGEs. Basic proteins can be separated repeatable and efficiently, allowing the analysis of protein impurities. The system is suitable for the analysis of antibodies with high pIs as demonstrated for monoclonal IgG_1 and llama antibodies. Using the PB-DS-PB coating, glycoforms and antibody binding products could be resolved in a repeatable way. Furthermore, the feasibility of using triple layer coated capillaries for CE-MS analysis was shown by the analysis of a llama antibody. Currently, we are carrying out a more in-depth evaluation of the performance and applicability of CE-MS using PB-DS-PB coatings. Such a system shows good potential for the efficient characterization of *e.g.* protein impurities, glycoforms and antibody binding products.

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Chapter 5

Capillary electrophoresis – mass spectrometry of intact basic proteins using Polybrene-dextran sulfate-Polybrene–coated capillaries: system optimization and performance

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Abstract

A capillary electrophoresis-mass spectrometry (CE-MS) method using sheath-liquid electrospray ionization interfacing was studied and optimized for the analysis of intact basic proteins. To prevent protein adsorption, capillaries with a noncovalent positively-charged coating were utilized. Capillaries were coated by subsequent rinsing with solutions of Polybrene, dextran sulfate and Polybrene. The coating proved to be fully compatible with MS detection, causing no background signals and ionization suppression. The composition of the sheath liquid and BGE were optimized using the model proteins α-chymotrypsinogen A, ribonuclease A, lysozyme and cytochrome *c*. A sheath-liquid of isopropanol-water-acetic acid (75:25:0.1, v/v/v) at 2 µL/min resulted in optimal signal intensities for most proteins, but caused dissociation or the heme group of cytochrome *c*. Optimum protein responses were obtained with a BGE of 50 mM acetic acid (pH 3.0), which allowed for baseline separation of the test proteins mixture. Several minor impurities present in the mixture could be detected and provisionally identified by using accurate mass and a protein modification database. The selectivity of the CE-MS system was investigated by the analysis of acetylated lysozyme. Eight highly related species, identified as nonacetylated lysozyme and lysozyme acetylated in various degrees, could be distinguished. The CE-MS system showed good reproducibility yielding interday (three week period) RSDs for migration time and peak area within 2% and 10%, respectively. With the CE-MS system, determination coefficients (R^2) for protein concentration and peak area were higher than 0.996, whereas detection limits were between 11 and 19 nM.

1. Introduction

In the fields of protein chemistry, biotechnology and biopharmaceutical development there is a demand for sensitive and selective analytical tools for the analysis of intact proteins. Capillary electrophoresis-mass spectrometry (CE-MS) combines high separation efficiency with the possibility of mass-selective detection and analyte characterization. With the maturing of the interfacing technology in the last decade, CE-MS has been increasingly used for relatively fast measurements of complex samples requiring great resolving power. CE-MS has been applied in various fields such as proteomics [1], metabolomics [2] and forensic science [3], as well as for pharmaceutical [4] and food analysis [5]. CE-MS also exhibits interesting possibilities for the characterization of intact proteins, providing information on protein quality including isoforms, degradation products and impurities [6]. Many protein modifications, like glycosylation, phosphorylation or deamidation, involve a change of net charge of a protein and thus also of its electrophoretic mobility. As CE has the intrinsic capacity to produce narrow peaks, it shows good potential for the separation of a variety of protein modifications in a single run.

MS detection of high mass accuracy and resolution, such as provided by time-of-flight (TOF) instruments, potentially provides highly useful information on the molecular weight of analyzed proteins species [7-9]. Coupling of CE and MS is most commonly carried out by electrospray ionization (ESI) using a sheath-liquid interface as it is robust and relatively easy to implement. Moreover, the sheath-liquid composition and flow rate can be specifically optimized to enhance MS signal intensities [10-12]. Sheath-liquid CE-MS of, for example, glycoproteins [13], biopharmaceuticals [14], metalloproteins [15] and dietary proteins [16] has been described.

A typical problem in protein CE is that the separation may be hampered by adsorption of proteins onto the fused-silica capillary wall [17, 18]. This may lead to fluctuations in the electroosmotic flow (EOF) and, thus, irreproducible migration times. Moreover, adsorption may cause severe band broadening and compromise the separation efficiency. In case of irreversible adsorption, the analyte recovery is affected and proper analyte quantification will be hindered. In order to allow efficient CE separations of proteins, coating of the inner capillary surface is often needed to minimize or completely prevent protein-wall interactions. To facilitate protein analysis by CE, over the past two decades, various types of capillary coatings have been developed [17-21]. Coatings can be either dynamic or static. Dynamic coating agents are continuously present in the BGE and reduce protein adsorption by interacting with the silica surface. Static coating materials are permanently attached to the capillary surface by incapillary silane chemistry and polymerization schemes, or by adsorption. The latter capillary coatings can be produced simply by flushing the capillary with solutions of an adsorptive coating agent.

Not all types of capillary coatings can be used when MS detection of proteins is pursued. In order to achieve efficient ESI-MS detection, coatings should be permanently

attached to the capillary wall to avoid background signals, suppression of analyte ionization, and/or contamination of the ion source and MS optics by coating agents. The coating should preferably be stable over time, allow multiple analyses, and use of different background electrolytes (BGEs) with respect to nature, concentration, and pH. Furthermore, a constant and appreciable EOF is preferred to achieve adequate and reproducible interfacing conditions. A very low or zero EOF may allow sheath liquid entering the separation capillary resulting in moving ion boundaries and pH shifts, which can compromise the CE separation [10]. Some researchers have advocated the application of extra pressure on the capillary inlet during CE-MS to induce a flow, however, hydrodynamic flow will reduce CE separation efficiencies.

An interesting and highly flexible approach, as introduced by Katayama *et al*. [22], is the use of successive multiple ionic polymer layers to construct stable adsorbed coatings. Previously, our group has shown the usefulness of capillaries that were noncovalently coated with a bilayer of Polybrene (PB) and poly(vinylsulfonic acid) (PVS) for the fast, reproducible and efficient separation of acidic proteins with CE-MS [14, 23]. However, this type of coating is not suitable for the analysis of basic proteins as the positively-charged proteins will adsorb to the negatively charged PVS layer. Therefore, a coating that exhibits a positively-charged outer layer would be more suitable [24-28]. In a recent study, we have demonstrated the suitability of a triple-layer capillary coating for the analysis of intact basic proteins by CE [29]. The coating was produced by successive flushing of the capillary with aqueous solutions of the charged polymers PB, dextran sulfate (DS) and PB. The coating effectively reduced protein adsorption [30] and allowed reproducible and efficient analysis of intact basic proteins using BGEs of Tris phosphate (pH 3-8). The feasibility of the PB-DS-PB coating for CE-MS was indicated briefly by the analysis of a llama antibody. However, no extensive optimization of interface parameters was carried out, and the overall performance of the CE-MS system employing PB-DS-PB coatings was not evaluated.

In this paper, CE-ESI-TOF-MS of intact basic proteins applying PB-DS-PB-coated capillaries is studied and optimized. The compatibility of the triple layer coating with ESI-MS is evaluated, and the sheath liquid and BGE composition were optimized with respect to protein MS response. The capability of the PB-DS-PB system to separate and characterize basic proteins is tested by using a mixture of model proteins, which also comprise some related impurities. The potential performance of the CE-MS system is further investigated by the analysis of acetylated lysozyme, representing a mixture of highly similar protein species. Finally, the CE-MS method is evaluated in terms of repeatability, response curves and limits of detection.

2. Materials and methods

2.1 Chemicals

Polybrene (hexadimethrine bromide, PB; average M_w 15,000), dextran sulfate (DS; average M_w > 500,000) sodium salt, N-methylmorpholine, isopropanol, acetonitrile and

methanol were purchased from Sigma-Aldrich (Steinheim, Germany). Triethylamine, acetic acid, formic acid and 25% (v/v) ammonium hydroxide were obtained from Merck (Darmstadt, Germany). The proteins α-chymotrypsinogen, ribonuclease A, cytochrome *c* and lysozyme were from Sigma-Aldrich. Protein test mixtures were prepared by diluting stock solutions (1 mg/mL) to the appropriate concentration with deionized water. Lysozyme was acetylated by adjusting 0.5 mL of an 0.1 mM aqueous solution of the protein to pH 10 using 0.1 M NaOH. Subsequently, 9 µL of 100 mM acetic anhydride in dioxane was added. The mixture was allowed to react for 30 min at room temperature before the sample was analyzed with CE-MS. Acetic acid BGEs were prepared by diluting acetic acid to the desired concentration with deionized water and adjusted to the appropriate pH with ammonium hydroxide, N-methylmorpholine, or triethylamine (each 1% in deionized water).

2.2 CE system

The experiments were carried out on a P/ACE MDQ capillary electrophoresis instrument (Beckman Coulter, Brea, CA, USA). Fused silica capillaries were from Polymicro Technologies (Phoenix, AZ, USA) having a total length of 80 cm and an internal diameter of 50 µm. Hydrodynamic injections were performed at 1 psi for 12 s (*i.e.* 1% of the total capillary volume). The separation voltage was -30 kV and the capillary temperature was 20 °C. New fused-silica capillaries were rinsed with 1 M NaOH for 30 min at 20 psi, and water for 15 min at 20 psi. After this treatment, capillaries were coated using the procedure described below.

2.3 Capillary coating

PB was dissolved in deionized water to a final concentration of 10% (w/v), and DS was dissolved to 0.5% (w/v) with deionized water. The coating agents were filtered over a 0.45 µm filter type HA (Millipore, Molsheim, France) prior to use. Capillaries were coated by subsequently rinsing 30 min with 10% (w/v) PB solution at 5 psi, 10 min with deionized water at 10 psi, 45 min with 0.5% (w/v) DS solution at 5 psi, 10 min with deionized water at 10 psi, 30 min with 10% (w/v) PB solution at 5 psi, and 10 min with deionized water at 10 psi. The capillary was then ready for CE analysis with the BGE of choice. Between runs, coated capillaries were flushed with BGE for 3 min at 10 psi. Overnight, capillaries were filled with BGE and tips were immersed in vials with BGE.

2.4 Mass spectrometry

MS was performed using a micrOTOF orthogonal-accelerated TOF mass spectrometer (Bruker Daltonics, Bremen, Germany). Source and transfer parameters were optimized by direct infusion of an ESI tuning mix (Agilent Technologies, Waldbronn, Germany). The mass spectrometer has a resolution of >15,000 FWHM in the mass range between *m/z* 1000-3000, as was determined from the tuning mix signals in this region. CE-MS coupling was realized

by a co-axial sheath-liquid interface (Agilent Technologies, Waldbronn, Germany). Sheath liquids were prepared by mixing the appropriate volumes of organic solvent (methanol, acetonitrile or isopropanol), deionized water and acidic component (formic acid or acetic acid). The sheath liquid was delivered by a 2.5 mL gas-tight syringe (Hamilton,Reno, NV, USA) using a syringe pump of Cole-Parmer (Vernon Hill, IL, USA). The following optimized spray conditions were used: dry gas temperature, 180 °C; nitrogen flow, 4 L/min; nebulizer pressure, 0.4 bar. Electrospray in positive ionization mode was achieved using an ESI voltage of -4.5 kV.

CE-MS data were analyzed using Bruker Daltonics Data Analysis software. Base-peak electropherograms (BPEs) were constructed for protein test mixtures in the range *m/z* 500-3000. Peak areas and plate numbers were determined from these BPEs. For determination of detection linearity and limit of detection (LOD), extracted-ion electropherograms (EIEs) were constructed for the respective proteins from their most abundant *m/z* signals. These were *m/z* 2139.0 and 2333.4 for α-chymotrypsinogen, *m/z* 1521.3 and 1711.2 for ribonuclease A, *m/z* 1431.5, 1590.3 and 1789.1 for lysozyme, and *m/z* 1236.8 and 1374.1 for cytochrome *c*. Protein ion charge assignment and molecular weight determinations were performed using the 'charge deconvolution' utility of the DataAnalysis software.

3. Results and discussion

3.1 Compatibility of PB-DS-PB coating with MS detection

From earlier experiments, we know that the PB-DS-PB coating effectively prevents protein adsorption of basic proteins [29, 30]. But when a capillary with a physically adsorbed coating like PB-DS-PB is combined with MS, it is important to establish potential interferences of the coating with the MS detection of proteins. The coating preferably should not cause background signals and/or ionization suppression. To investigate these compatibility aspects, first a BGE of 175 mM acetic acid (pH 2.7) was infused through a PB-DS-PB-coated capillary into the sheath-liquid interface applying standard interface and MS conditions. No specific signals originating from the coating agents PB and DS could be observed in the recorded mass spectra. As a control experiment, also 175 mM acetic acid containing 1 mg/mL PB was infused, yielding several intense and distinct signals related to PB. To check potential protein ionization suppression by PB, solutions of the individual test proteins ribonuclease A and lysozyme (50 µg/mL) in 175 mM acetic acid comprising no or increasing concentrations of PB (100 pg/mL - 1 mg/mL) were led through a PB-DS-PB coated capillary to the CE-MS interface. Under these conditions the proteins exhibit a net positive charge and adsorption to the positively-charged capillary wall will be effectively avoided. For the protein solutions without PB highest protein intensities were obtained and (again) no PB signals were observed in the mass spectra. Addition of very low PB concentrations (1 ng/mL) did not affect the protein signals. However, above 10 ng/mL

(ribonuclease A) or 1 µg/mL (lysozyme) a clear decrease of the protein signal intensities was observed, most probably due to ionization suppression by PB. From these experiments it follows that the PB present in the triple layer coating does not cause any measurable ionization suppression.

3.2 Optimization of sheath-liquid

The sheath liquid not only serves to make the electrical contact required for CE, it also provides the conditions for ESI. So, optimization of the composition and flow rate of the sheath liquid is essential to achieve good MS responses for proteins. As starting point, a sheath liquid was used of organic solvent-water (1:1, v/v) containing 1% (v/v) of an organic acid to provide conductivity and promote positive ionization. Methanol, acetonitrile and isopropanol were tested as organic solvent and either acetic acid or formic acid was added. Applying the various sheath liquids, the basic model proteins, α-chymotrypsinogen A, ribonuclease A, cytochrome *c* and lysozyme (50 µg/mL each), were individually analyzed by CE-MS using a PB-DS-PB coated capillary and a BGE of 175 mM acetic acid (pH 2.7). Figure 5.1 shows the relative peak area as obtained for ribonuclease A; the relative peak area of the other proteins showed the same trend. A sheath liquid comprising isopropanol resulted in the most intense protein signals; sheath liquids containing methanol and acetonitrile yielded considerable lower signals (factor 2.5-3). In all sheath liquids, acetic acid appeared to enhance protein ionization stronger than formic acid (factor 2-3). Signal to noise (S/N) ratio of the protein peaks demonstrated similar optima. So, isopropanol and acetic acid were chosen for further experiments. Selecting 1% (v/v) acetic acid, the concentration isopropanol of the sheath liquid was varied. CE-MS of ribonuclease A showed that a sheath liquid of 75% (v/v) isopropanol provided the highest protein peak area (Figure 5.2A). The same optimal isopropanol concentration was found for α-chymotrypsinogen and lysozyme, however, for cytochrome *c* the optimal protein signals were obtained with a sheath liquid comprising 25%

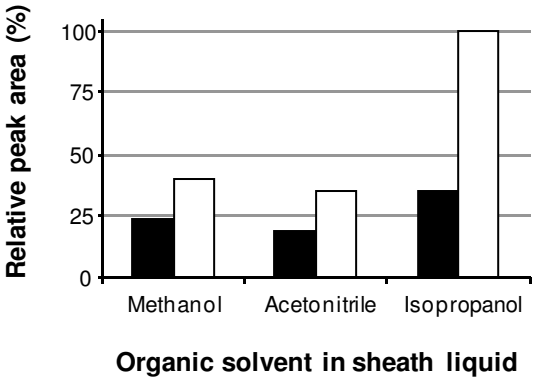

Figure 5.1. Influence of organic solvent and acidic component (black bar, formic acid; white bar, acetic acid) in the sheath liquid on the relative peak area obtained during CE-MS of ribonuclease A (50 µg/mL). Conditions: BGE, 175 mM acetic acid (pH 2.7); sheath liquid, organic solvent-water-acid (50:50:1, v/v/v) at 2 µL/min. Further conditions, see Experimental Section.

(v/v) isopropanol. The mass spectrum of cytochrome *c* obtained with a sheath liquid containing 75% (v/v) isopropanol showed an intense signal at *m/z* 617.2. This signal originates from the heme-group that dissociates from the protein upon ESI [31, 32]. Apparently, the gas phase dissociation of the heme group is promoted by the high concentration of organic solvent as was also found by others during ESI-MS infusion experiments of cytochrome *c* [33-35].

The acetic acid content of a sheath liquid of isopropanol-water (75:25, v/v) was varied between 0 and 5% (v/v), and the peak area of ribonuclease A was monitored (Figure 5.2B). A small increase in protein peak area was observed between 0 and 0.1% (v/v), which can be attributed to enhanced protonation of the protein during ESI. Acetic acid concentrations above 0.1% (v/v), however, decreased the protein signal, most probably due to increased ionization suppression [12]. So, the acetic acid in the sheath liquid has a dual effect leading to an optimal concentration of 0.1% (v/v). The same optimum was found for the other proteins.

Finally, the flow rate of the sheath liquid was varied between 0.5 and 5 µL/min applying a sheath liquid of isopropanol-water-acetic acid (75:25:0.1, v/v/v) (Figure 5.2C). As depicted for ribonuclease A, between 0.5 and 2 µL/min a significant increase in protein peak area was observed. At low flow rates the electrospray formation was less efficient, as was also reflected by unstable baselines and periodic loss of signal during CE-MS. At a sheath liquid flow rate above 2 µL/min the dilution effect of the sheath liquid becomes eminent, decreasing the protein ESI signal which is predominantly concentration sensitive. A sheath-liquid flow rate of 2 µL/min provided stable signals and was selected for further experiments.

3.3 Optimization of BGE

In a preliminary study we found acetic acid to be an adequate BGE for CE-MS of proteins using PB-DS-PB-coated capillaries [29]. As acetic acid proved to be an appropriate

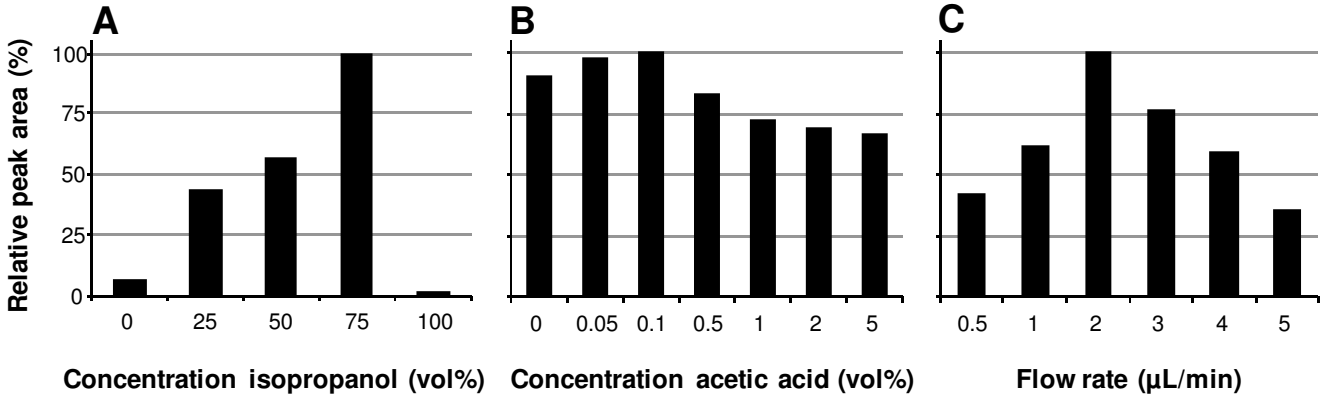

Figure 5.2. Influence of (A) the isopropanol concentration, (B) the acetic acid concentration and (C) the flow rate of the sheath liquid on the relative peak area obtained during CE-MS of ribonuclease A (50 µg/mL). Conditions: BGE, 175 mM acetic acid (pH 2.7); sheath liquid, (A) various ratios isopropanol-water containing 1% (v/v) acetic acid; (B) isopropanol-water (75:25, v/v) containing various percentages acetic acid; (C) isopropanol-water-acetic acid (75:25:0.1, v/v/v). Sheath-liquid flow rate, (A and B) 2 µL/min. Further conditions, see Experimental Section.

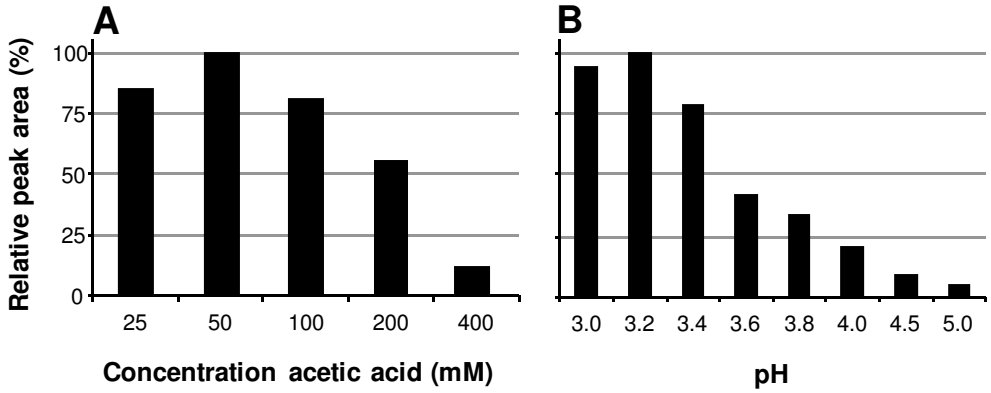

Figure 5.3. Influence of (A) the concentration acetic acid in the BGE and (B) the pH of the BGE on the relative peak area obtained during CE-MS of ribonuclease A (50 µg/mL). Conditions: BGE, (A) various concentration acetic acid (pH 3.0); (B) 50 mM acetic acid of various pH; sheath liquid, isopropanol-water-acetic acid (75:25:0.1, v/v/v) at 2 µL/min. Further conditions, see Experimental Section.

sheath-liquid additive, we decided to select acetic acid as BGE and optimize its composition with respect to protein MS signal intensity. Acetic acid BGEs with concentrations between 25 and 400 mM, which were all adjusted to pH 3.0 using ammonium hydroxide, were tested by analyzing the test proteins by CE-MS using a triple layer coated capillary. As depicted for ribonuclease A (Figure 5.3A), a slight increase in protein peak area is obtained going from 25 to 50 mM acetic acid in the BGE. When applying acetic acid concentrations above 50 mM the protein peak area steadily decreased. The same trend was observed for the other test proteins. Selecting a BGE of 50 mM acetic acid and increasing its pH up to 5.0 by adjustment with ammonium hydroxide also led to decreasing protein peak areas (Figure 5.3B). Most probably, the decrement in peak area as observed for both the higher BGE concentrations and pH values is mainly caused by the increasing amount of ammonium ions present in the respective BGEs, which causes ionization suppression of the proteins [12]. Therefore, as alternative N-methylmorpholine and triethylamine were also considered as basic compounds to adjust the pH of the acetic acid BGE. However, these components appeared to cause even more ionization suppression than ammonium hydroxide. For instance, BGEs of 50 mM acetic acid adjusted with N-methylmorpholine or triethylamine to pH 3.0 gave peak areas for ribonuclease A that were, respectively, a factor 2.5 and 5.5 lower than when ammonium hydroxide was used.

Obviously, the BGE should also be optimized with respect to the separation performance. Using a BGE of 25 mM acetic acid (pH 3.0) a baseline separation of the four proteins was obtained. Raising the acetic acid concentration up to 400 mM, protein migration times increased up to a factor of three – mostly due to a reduced EOF – while the separation was maintained. Increase of the pH of the BGE also caused migration times to increase. Moreover, in the pH 3.2-3.8 region, lysozyme, cytochrome *c* and their impurities (see below) could not be fully separated, which is in agreement with previous results [29]. Considering the

effect of the BGE on both the separation and protein peak areas, a BGE of 50 mM acetic acid adjusted with ammonium hydroxide to pH 3.0 was selected for further CE-MS.

3.4 CE-MS of basic proteins

Performing CE-MS with a BGE of 50 mM acetic acid (pH 3.0) in combination with a sheath-liquid of isopropanol-water-acetic acid (75:25:0.1, v/v/v), a baseline separation of the basic test proteins with narrow and quite symmetrical electrophoretic peaks was obtained within 12 min (Figure 5.4A). Typical plate numbers were between 70,000 and 100,000, which is rather favorable for CE-MS of proteins. Good quality mass spectra were obtained (Figure 5.4B) allowing the assignment of the molecular masses of the respective proteins (Table 5.1). Deconvolution of the mass spectra yielded masses of 25656.3 Da (α-chymotrypsinogen), 13680.9 Da (ribonuclease A), 14304.0 Da (lysozyme), and 12357.7 Da (cytochrome *c*), respectively, which agreed well with the expected molecular masses. The ESI mass spectra of α-chymotrypsinogen A, ribonuclease A, and lysozyme are dominated by charge states (12+, 9+, and 9+, respectively) which are in agreement with the theoretical average charge state of the non-denatured protein in the gas phase (12.5+, 9.1+, and 9.3+, respectively) [36]. These values indicate that the proteins did not unfold during ESI. As mentioned before, under the applied conditions an intense signal for the heme group (*m/z* 617.2) is observed in the mass spectrum of cytochrome *c*. Interestingly, the charge envelope of cytochrome *c* in this spectrum is shifted to lower *m/z* values indicating a higher average charge than is theoretically expected (13+ vs. 8.7+, respectively). Apparently, the loss of the heme group during ESI results in a denaturation of the protein. When CE-MS analysis of the protein mixture was performed using a sheath liquid containing 25% (v/v) isopropanol, the average charge state decreased to 9+ whereas no signal for the heme group is observed. So, with sheath liquids containing lower percentages of isopropanol, cytochrome *c* does not denature during ESI and retains its heme group.

CE-MS analysis of the protein mixture revealed several extra peaks, most probably caused by protein degradation/modification (Figure 5.4A). By CE-MS analysis of the individual proteins it was known to which protein each impurity was related. Good quality mass spectra were obtained for all protein impurities and deconvoluted masses could be determined (Table 5.1). Provisional assignment of the minor compounds was based on the deconvoluted mass and considering protein modifications as listed in the Unimod database [37]. CE-MS is especially suitable to reveal protein modifications resulting in change of charge with respect to the parent protein. Deamidations, as were observed for ribonuclease A, lysozyme and cytochrome *c*, result in a very small change of molecular weight (+1 or +2 Da), but also in a decrease of the net positive charge of the protein and, thus, an effective CE separation. Methylation of an carboxylic acid group or the substitution of asparagine for lysine, as found as potential modification of α-chymotrypsinogen (Table 5.1), may result in a net gain of positive charge causing the impurity to migrate after the parent protein peak. The

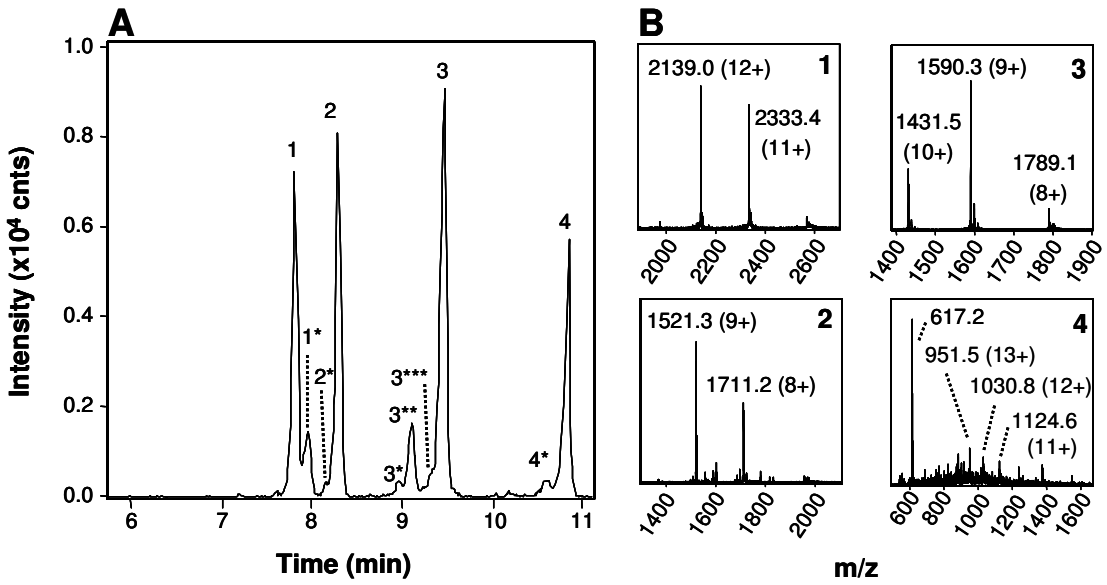

Figure 5.4. (A) CE-TOF-MS of a mixture of (1) α-chymotrypsinogen A, (2) ribonuclease A, (3) lysozyme and (4) cytochrome *c* (50 µg/mL each) using a PB-DS-PB coated capillary. (B) Mass spectra obtained for peaks 1, 2, 3 and 4, respectively. The *m/z* values and charge states are given for the main peaks. Conditions: BGE, 50 mM acetic acid (pH 3.0); sheath-liquid, isopropanol-water-acetic acid (75:25:0.1, v/v/v) at 2 µL/min. Further conditions, see Experimental Section.

Table 5.1. Deconvoluted masses and provisional identification of the peaks observed during CE-TOF-MS of the protein test mixture.

Peak no.	Deconvoluted mass	Assignment	Interpretation
1	25656.3	M	α-Chymotrypsinogen
1*	25669.9	M + 14	Methylated α-chymotrypsinogen or lysine for asparagine substitution
2	13680.9	M	Ribonuclease A
2*	13681.8	M + 1	Deamidated ribonuclease A
3	14304.0	M	Lysozyme
3*	14306.0	M + 2	Bisdeamidated lysozyme
3**	14304.0	M + 0	Unknown modification of lysozyme
3***	14466.1	M + 162	Hexose addition to lysozyme
4	12357.7	M	Cytochrome *c*
4*	12359.7	M + 2	Bisdeamidated cytochrome *c*

addition of a hexose group, found as impurity of lysozyme, does not induce a charge difference within the protein, however, a partial separation from the main peak is still realized. Apparently, the increase in molecular weight (+162 Da) is the main contributor to the separation by lowering the effective mobility of the protein. Interestingly, the major impurity of lysozyme has a molecular weight similar to lysozyme. An assignment could not be derived for this impurity, however, as it migrated before the main peak it has a net higher positive charge. Notably, this impurity, having the same molecular weight as lysozyme, would

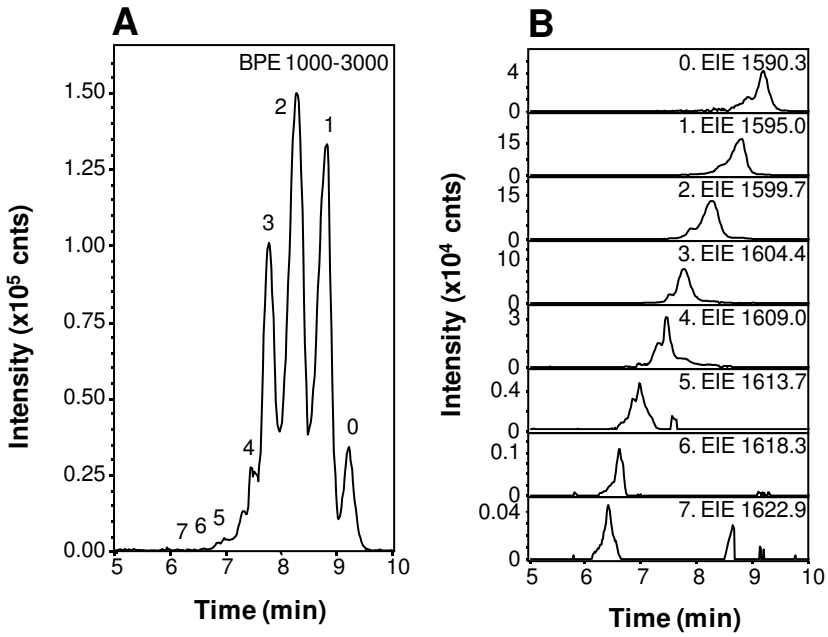

Figure 5.5. (A) CE-TOF-MS of acetylated lysozyme using a PB-DS-PB coated capillary. (B) EIEs for the unmodified lysozyme (0) and lysozyme containing 1-7 acetylation, respectively (1-7). Injection, 4 psi, 12 s; Other conditions, see Figure 5.4.

not have been detected if it would not have been separated from the main peak.

In order to further test the capability of the CE-MS system using PB-DS-PB coated capillaries to distinguish highly related proteins, a test mixture was prepared by acetylation of lysozyme with acetic anhydride. Acetylation of primary amine groups in the protein (*i.e.*, six lysine ε-amino groups and one N-terminal α-amino group) leads per acetylation to a net reduction of one positive charge and a gain in molecular weight of 42 Da. The resulting mixture of acetylated products was analyzed by CE-MS with the PB-DS-PB system using a BGE of 50 mM acetic acid (pH 3.0). The BPE nicely showed eight peaks (Figure 5.5A); for all eight species EIEs could be constructed (Figure 5.5B). Deconvolution of the respective mass spectra revealed the masses of the parent lysozyme (peak 0) and of lysozyme acetylated in various degrees (peak 1 to 7, respectively). The results indicate that all available amino groups are prone to acetylation. The applied reaction conditions lead to a triple-acetylated lysozyme as main reaction product, but non-acetylated lysozyme was still present, whereas also a fraction of fully acetylated lysozyme was formed. The ESI mass spectra of lysozyme and its acetylated products were all dominated by the $(M+9H)^{9+}$ ion. So, the acetylation did not cause unfolding of the protein, and did not affect the average charge state of the protein in the gas phase [38, 39]. Closer inspection of the EIEs shows a consistent sub-peak in front of the main peaks. This minor peak probably originates from the main impurity of lysozyme (see above), which apparently shows similar susceptibility towards acetylation as lysozyme. Overall, the results obtained for this acetylated lysozyme sample clearly demonstrate the

capability of the CE-TOF-MS system using PB-DS-PB-coated capillaries to differentiate between highly related and modified protein species.

3.5 Method evaluation

To further evaluate the analytical performance of the developed CE-MS method, repeatability, response curves and LODs were determined. The repeatability of the method was examined by successively (n=40) analyzing the same model protein mixture by CE-MS over a period of 16 h. Between runs the PB-DS-PB coated capillary was only flushed with BGE. Migration time RSDs were less than 1.6%, whereas peak area RSDs were within 9% (Table 5.2). The PB-DS-PB coating also showed excellent long-term stability. During three consecutive weeks and on three days per week, lysozyme (50 µg/mL) was analyzed five times by CE-MS. Each day a fresh sample was prepared, while the same PB-DS-PB coated capillary was used for all analyses and, again, no in-between regeneration was carried out. The migration time RSD (n=45) over the entire three weeks was below 1.8%, whereas the peak area RSD of lysozyme was within 10%. Response curves for all test proteins were established by analyzing protein concentrations between 1 and 250 µg/mL in triplicate by CE-MS. For all proteins, determination coefficients (R^2) above 0.996 were obtained over the entire concentration range (Table 5.3). LODs (S/N 3) ranged from 11 nM for ribonuclease A up to 19 nM for lysozyme. To our knowledge, such favorable LODs have not been reported before using sheath-liquid CE-MS analysis of intact proteins.

Table 5.2. Intraday (n=40) RSDs of the migration time and peak area of the test proteins obtained during repeated CE-TOF-MS analyses using a PB-DS-PB coated capillary. Conditions: BGE, 50 mM acetic acid (pH 3.0); sheath-liquid, isopropanol-water-acetic acid (75:25:0.1, v/v/v) at 2 µL/min. Further conditions, see Experimental Section.

Protein	Migration time RSD (%)	Peak area RSD (%)
α-Chymotrypsinogen	1.2	7.3
Ribonuclease A	1.0	6.6
Lysozyme	1.4	8.3
Cytochrome *c*	1.6	8.0

Table 5.3. Determination coefficients and detection limits for four model proteins obtained during CE-MS using a PB-DS-PB coated capillary and a BGE of 50 mM acetic acid (pH 3.0).

Protein [a]	Determination coefficient (R^2)	LOD (nM) [b]
α-Chymotrypsinogen	0.996	16
Ribonuclease A	0.998	11
Lysozyme	0.999	19
Cytochrome *c*[c]	0.998	14

a. Sheath liquid, isopropanol-water-acetic acid (75:25:0.1, v/v/v), except for cytochrome *c*, (25:75:0.1, v/v/v).

b. Concentration to yield a S/N ratio of 3 as extrapolated from a 3.3-µg/mL injection.

4. Conclusions

In this work, the performance of PB-DS-PB coated capillaries for the CE-ESI-TOF-MS analysis of intact basic proteins was optimized and evaluated. The preparation of the coating is simple and fast comprising only flushes of the capillary with solutions of the coating agents. The coating shows full compatibility with MS detection, causing neither background signals nor ionization suppression. Fast and efficient protein separations with optimal protein signals were obtained after optimization of both the BGE and sheath-liquid composition. As demonstrated by the gas phase dissociation of the heme group from cytochrome *c* and unfolding of the protein at higher isopropanol concentrations in the sheath liquid, the sheath liquid may induce changes in protein conformation. The developed CE-MS system appeared to be especially suited for the characterization of protein modifications that lead to charge difference, as was demonstrated by the identification of deamidated products in the model proteins. Accordingly, acetylation of the lysine amino groups in lysozyme resulted in a mixture of highly similar products that were separated efficiently with CE using the triple layer coating. Evaluation of the CE-MS method showed that favorable RSD values for both migration times and protein peak areas, as well as very low-nM LODs were obtained. Overall, it can be concluded that CE-ESI-TOF-MS using PB-DS-DS coatings is very useful for intact protein analysis. Currently, we are investigating the potential of this system for the profiling of biopharmaceuticals, such as oxytocin and interferon-β.

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Chapter 6

The analysis of biopharmaceuticals by capillary electrophoresis – mass spectrometry using noncovalently coated capillaries

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Abstract

In this work, the usefulness of capillary electrophoresis – electrospray ionization time-of-flight–mass spectrometry (CE-ESI-TOF-MS) for the analysis of biopharmaceuticals was studied. Noncovalently bound capillary coatings consisting of a bilayer of Polybrene-poly(vinylsulfonic acid) (PB-PVS) or a triple layer of Polybrene-dextran sulfate-Polybrene (PB-DS-PB) were used to minimize protein and peptide adsorption, and achieve good separation efficiencies. The potential of the CE-MS systems to characterize degradation products was investigated analyzing samples of the drugs recombinant human growth hormone (rhGH) and oxytocin, which had been subjected to prolonged storage, heat exposure and/or different pH values. Modifications of rhGH and oxytocin, such as deamidations, oxidations, sulfonate formation and sulfur additions, could be assigned based on the CE separation and accurate masses as obtained for the components of the degraded samples. Recombinant human interferon β-1a (rhIFN-β) was used to evaluate the capability of the CE-MS method to assess glycan heterogeneity of pharmaceutical proteins. Analysis of this N-glycosylated protein revealed a cluster of resolved peaks which appeared to be caused by at least ten glycoforms differing merely in sialic acid and hexose N-acetylhexosamine composition. Based on the relative peak area (assuming an equal molar detector response per glycoform), a quantitative glycoform profile could be derived which may show useful for in-process and quality control of rhIFN-β batches. It is concluded that the separation power provided by combined CE and TOF-MS allows discrimination of highly related protein species.

1. Introduction

With efficient methodologies available in biotechnology today, increasing numbers of recombinantly manufactured pharmaceutical peptides and proteins are being commercialized [1,2]. The assessment of biopharmaceutical quality in terms of identity, content and purity is an important issue during manufacturing. The biotechnological production process may show variability, which can introduce product diversity, isoforms and closely-related degradation products [3,4]. With the appearance of biosimilars, *i.e.* attempted copies of the original biopharmaceutical [4], the demand for extensive characterization of therapeutic proteins and peptides has even further increased. The variability and stability between biosimilar and originator product might differ even more significantly than between batches of the same product. Clearly, there is an increasing demand for suitable analytical methods that allow not only protein and peptide identification, but also the separation and quantification of impurities and possible degradation products.

Capillary electrophoresis-mass spectrometry (CE-MS) provides the high separation efficiency and mass-selective detection [5,6] that can be very useful for biopharmaceutical product characterization. Many protein modifications, like glycosylation and deamidation, may involve changes of net charge of a protein and, thus, also of its electrophoretic mobility. As CE has the intrinsic capacity to produce narrow peaks, it shows good potential for the separation of a variety of protein modifications in a single run. MS detection of high mass accuracy and resolution, such as provided by time-of-flight (TOF) instruments can yield accurate information on the molecular weight of analyzed proteins species. CE-MS is most commonly carried out applying electrospray ionization (ESI) using a sheath-liquid interface, which is relatively robust and easy to implement. The sheath-liquid composition and flow rate can be optimized to enhance protein signal intensities [7,8]. So far, the application of sheath-liquid CE-MS for the analysis of biopharmaceuticals has been quite limited. Staub *et al.* developed a CE-TOF-MS method to distinguish natural human growth hormone (hGH) from recombinant hGH [9], and Catai *et al.* showed the feasibility of CE-ion trap-MS for the determination of some degradation products in hGH after heat exposure and prolonged storage [10,11]. Neusüß *et al.* and Sanz-Nebot *et al.* demonstrated the usefulness of CE-TOF-MS for the glycoforms characterization of erythropoietin [12-16].

The tendency of protein molecules to adsorb to fused-silica may result in deterioration of CE separation efficiencies. In order to prevent adverse interactions, it is a common strategy to coat the internal wall surfaces with agents that minimize protein adsorption [17,18]. Two general coating strategies can be discerned in CE: dynamic and static coating [19]. Dynamic coating agents are continuously present in the BGE and reduce protein adsorption by reversibly binding to adsorption sites on the silica surface. For CE-MS, a disadvantage of this type of coating is that coating agents may enter the ion source and, thereby, can result in ionization suppression of the analyte and contamination of the mass spectrometer. In order to prevent MS detection interferences, static coatings have to be used. These coatings are

permanently attached to the capillary surface by adsorption or by covalent coating involving silane chemistry and polymerization schemes. The former capillary coatings can be produced simply by flushing the capillary with solutions of highly adsorptive agents. Very stable and uniform adsorbed coatings can be produced by applying multiple layers of oppositely charged polymers, as indicated for the first time by Katayama *et al*. [20,21], and demonstrated by others subsequently [22-29].

Over the last years, we have studied and shown the usefulness of a bilayer coating of Polybrene-poly(vinylsulfonic acid) (PB-PVS) and a triple layer coating of Polybrene-dextran sulfate-Polybrene (PB-DS-PB) for the CE-UV and CE-MS analysis of peptides and proteins [10,11,30-33]. The negatively-charged PB-PVS coating was used in combination with a background electrolyte (BGE) of medium or high pH for the analysis of acidic peptides and proteins [10,11,30,31], whereas for basic proteins the positively-charged PB-DS-PB coating was used in combination with a low-pH BGE [32,33]. The multilayer coatings exhibit a pH-independent and highly reproducible EOF, and effectively prevent protein adsorption [34], resulting in efficient protein separations. In addition, these coatings show very good compatibility with ESI-MS detection [31,33].

In the study presented here, the performance and usefulness of CE-MS systems employing PB-PVS and PB-DS-PB coated capillaries for the characterization of biopharmaceuticals was investigated. Representative samples of the drugs recombinant human growth hormone (rhGH, 22 kDa, p*I* 5.1), recombinant human interferon-β-1a (rhIFN-β, 23 kDa, pI 9.6) and oxytocin (1 kDa, p*I* 8.6) were used for testing. The potential of the CE-MS systems to separate and identify degradation products resulting from heat stress and/or prolonged storage of rhGH and oxytocin was studied. In addition, rhIFN-β was analyzed to evaluate the capability of the PB-DS-PB CE-TOF-MS method to assess the glycoform heterogeneity of a pharmaceutical protein. Some attention was paid to optimization of separation and detection conditions, but emphasis was on the type of information on the sample composition that can be obtained with CE-TOF-MS.

2. Materials and methods

2.1 Chemicals

Polybrene (hexadimethrine bromide, PB; average M_w 15,000), dextran sulfate (DS; average M_w > 500,000) sodium salt, 25% (w/v) poly (vinylsulfonic acid) (PVS; average M_w 4000-6000), isopropanol and acetonitrile were purchased from Sigma-Aldrich (Steinheim, Germany). Acetic acid, formic acid and 25% (v/v) ammonium hydroxide were obtained from Merck (Darmstadt, Germany). The ammonium formate BGE was made by diluting ammonium hydroxide to 75 mM and adjusting the pH to 8.5 with 1% (v/v) formic acid in deionized water. Acetic acid BGEs were prepared by diluting acetic acid to the desired

concentration with deionized water and adjusted to the appropriate pH with 1% (v/v) ammonium hydroxide in deionized water.

A solution of rhGH had been prepared previously by dissolving somatropin CRS (European Directorate for the Quality of Medicines, Strasbourg, France) in 30 mM Tris phosphate (pH 7.5) to a concentration of 1.5 mg/mL. An aliquot of this sample was incubated at 40°C for 24 h and subsequently had been stored for 12 months at -18°C. Oxytocin acetate powder (Sigma Aldrich, Zwijndrecht, the Netherlands) had been dissolved previously in 50 mM phosphate buffer with a pH of 2.0, 4.5, 7.0, and 9.0 to a final concentration of 50 µg/mL. Aliquots of these solutions had been subsequently exposed to 70°C for 64 h and then were stored at 4°C until analysis. Bulk rhIFN-β-1a was supplied by Biogen Idec Inc. (Cambridge, MA, USA) as a 0.27 mg/mL solution in 100 mM sodium phosphate buffer and 200 mM sodium chloride at pH 7.2. Reformulated rhIFN β-1a was produced by dialysis of bulk rhIFN-β-1a with a 3.5 kDa molecular weight cut-off (MWCO) Slide-A-Lyzer Cassette (Perbio Science, Etten-Leur, the Netherlands) against a commercially used formulation containing 20 mM sodium acetate buffer, 150 mM L-arginine monohydrochloride and 0.04 mM Tween 20 (Sigma Aldrich, Zwijndrecht, the Netherlands) at pH 4.8, and subsequent filtration through a 0.22 µm polyethersulfone membrane (Millipore, Amsterdam, the Netherlands). This solution was stored at 4°C until analysis. Prior to CE-MS analysis the formulation buffer was exchanged for water using a MWCO filter of 3 kDa. The final solution had a rhIFN-β concentration of 450 µg/mL.

2.2 CE system

The experiments were carried out on a P/ACE MDQ capillary electrophoresis instrument (Beckman Coulter, Brea, CA, USA). Fused silica capillaries were from Polymicro Technologies (Phoenix, AZ, USA) having a total length of 80 cm and an internal diameter of 50 µm. Hydrodynamic injections were performed at 1 psi for 12 s (*i.e.* 1% of the total capillary volume). The separation voltage was 30 kV (PB-PVS coating) or -30 kV (PB-DS-PB coating) and the capillary temperature was 20 °C. New fused-silica capillaries were rinsed with 1 M NaOH for 30 min at 20 psi, and water for 15 min at 20 psi. After this treatment, capillaries were coated with either a PB-PVS coating or a PB-DS-PB coating using the procedures described below.

2.3 Capillary coating

PB was dissolved in deionized water to a final concentration of 10 % (w/v), and DS was dissolved to 0.5 % (w/v) with deionized water. PVS was diluted in deionized water to 1 % (w/v). The coating agents were filtered over a 0.45 µm filter type HA (Millipore, Molsheim, France) prior to use. The PB-PVS coating was applied by subsequently rinsing 30 min with 10% (w/v) PB solution at 5 psi, 10 min with deionized water at 10 psi, 30 min with 1% (v/v) PVS solution at 5 psi, and 10 min with deionized water at 10 psi. The PB-DS-PB

coating was applied by subsequently rinsing 30 min with 10% (w/v) PB solution at 5 psi, 10 min with deionized water at 10 psi, 45 min with 0.5% (w/v) DS solution at 5 psi, 10 min with deionized water at 10 psi, 30 min with 10% (w/v) PB solution at 5 psi. After the final coating agent flushing step, the capillary was rinsed 10 min with deionized water at 10 psi. The capillary was then ready for CE analysis with the BGE of choice. Between runs, coated capillaries were flushed with BGE for 3 min at 10 psi. Overnight, capillaries were filled with BGE and tips were immersed in vials with BGE.

2.4 Mass spectrometry

MS was performed using a micrOTOF orthogonal-accelerated TOF mass spectrometer (Bruker Daltonics, Bremen, Germany). Source and transfer parameters were optimized by direct infusion of an ESI tuning mix (Agilent Technologies, Waldbronn, Germany). CE-MS coupling was realized by a co-axial sheath-liquid interface (Agilent Technologies, Waldbronn, Germany). For the analysis of rhGH the sheath liquid was acetonitrile-water-formic acid (75/25/5, v/v/v) at a flow rate of 4 µL/min. Sheath liquids (2 µL/min) for oxytocin and rhIFN-β analyses were isopropanol-water-acetic acid (75/25/0.1, v/v/v) and isopropanol-water-acetic acid (25/75/0.5, v/v/v), respectively. Sheath liquid was delivered by a 2.5 mL gas-tight syringe (Hamilton, Reno, NV, USA) using a syringe pump of Cole-Parmer (Vernon Hill, IL, USA). The following optimized spray conditions were used: dry gas temperature, 180 °C; nitrogen flow, 4 L/min; nebulizer pressure, 0.4 bar. Electrospray in positive ionization mode was achieved using an ESI voltage of -4.5 kV. To assure proper ion transfer, the analysis of rhGH and rh IFN-β were performed with a capillary exit and skimmer voltage of 250 V and 83 V, respectively. For the analysis of oxytocin, these voltages were set at to 200 V and 50 V, respectively. CE-MS data were analyzed using Bruker Daltonics Data Analysis software. Molecular weight determinations of proteins were performed using the 'Charge Deconvolution' utility of the DataAnalysis software.

3. Results and discussion

3.1 Recombinant human growth hormone

rhGH is a therapeutic protein used in the treatment of retarded growth and dwarfism caused by the inadequate production of the hormone during the growth period. The monograph of rhGH in the European Pharmacopeoia includes a CE method using bare fused-silica capillaries for detection of charge variants [35]. Catai *et al*. proposed the use of PB-PVS coated capillaries for rhGH analysis by CE in order to improve the method's repeatability, resolution and efficiency [10]. Furthermore, an MS-compatible method was developed using a volatile BGE of ammonium formate [10,11]. However, the resolution and mass accuracy of the used ion trap mass spectrometer were not sufficient to unambiguously assign rhGH

degradation products. Here we study the potential of CE-TOF-MS with PB-PVS coated capillaries for the characterization of a heat exposed and stored rhGH sample.

First, a solution of 1.5 mg/mL rhGH (somatropin CRS) was analyzed by CE-TOF-MS using a BGE of 75 ammonium formate (pH 8.5). Under these conditions both the protein (p*I* 5.1) and the PB-PVS coated capillary wall are negatively charged, preventing protein adsorption. A sheath liquid of low pH was used to allow efficient positive ESI. A narrow symmetric peak (N, 105,000) was obtained for rhGH at a migration time of approximately 11.5 min (Figure 6.1A, peak 0). Deconvolution of the mass spectrum obtained in the apex of the peak (Figure 6.1B) revealed a protein molecular weight of 22123.9 Da, which is in well agreement with the expected molecular weight for rhGH. Remarkably, the mass spectrum seems to comprise two charge state distributions. This might be explained by partial unfolding of the protein during ESI, which leads to increased charging and a shift of the distribution to lower *m/z* values [36]. Next to hGH, a small additional peak at 10.2 min was detected. Its mass spectrum shows major signals at *m/z* 804.3 and 986.4, but no typical protein charge state distribution. The sample component could not be identified but we presume it is a low-molecular weight compound. Next, an aqueous solution of rhGH CRS (1.5 mg/mL) that was exposed to heat (40°C) for 24 h and then had been stored for one year at -18°C was analyzed using the PB-PVS CE-TOF-MS system. The resulting base peak electropherogram (BPE) shows that several degradation products have been formed (Figure 6.1C). The unknown impurity was again observed at 10.2 min. The protein mass spectra obtained for the peaks between 11.5 and 13.0 min are depicted in Figure 6.1D, yielding deconvoluted masses of 22156.1, 22157.0, 22252.3, and 22253.3 Da for peaks 1 to 4, respectively. Remarkably, no unmodified rhGH (22124 Da) was found in the sample, although the main degradation product (peak 1) had virtually the same migration time as rhGH CRS (11.6 min). The observed mass difference of 32 Da for peak 1 with respect to rhGH CRS suggests the main degradation product to be doubly oxidized rhGH. Oxidation of methionine residues is a common modification observed for rhGH [37]. The gain of two oxygen atoms will hardly affect the electrophoretic mobility of the protein, as the oxidation induces no change in the protein charge and only a very small relative change in protein mass. The compound migrating at 11.8 min (peak 2) is clearly separated from the oxidized rhGH (peak 1) and differs only 0.9 Da in mass. Most probably, peak 2 is caused by rhGH that is oxidized (twice) as well as deamidated (once). Deamidation is a common protein degradation [38] that results in a small change of protein molecular mass (+0.984 Da), but at pH 8.5 also in a net increase of one negative charge of the protein due to the formation of an aspartic acid residue. As a consequence, the protein's electrophoretic mobility changes significantly. The molecular mass of the degradation product migrating at 12.2 min (peak 3) is 128 Da higher than rhGH CRS. As this compound migrates after the deamidated product (peak 2) it most likely has gained at least two negative charges with respect to rhGH. Therefore, the mass difference of 128 Da

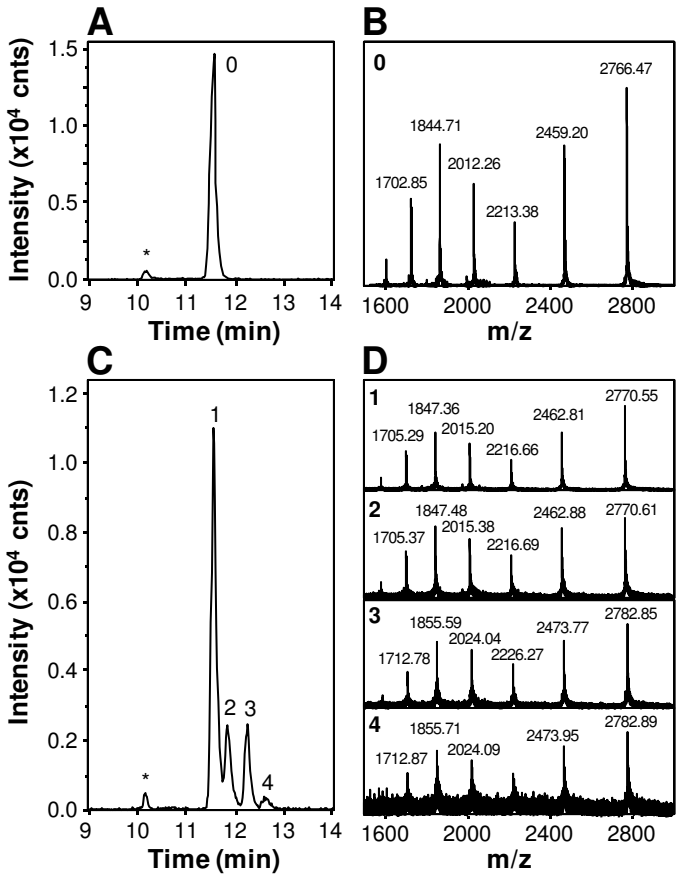


Figure 6.1. CE-TOF-MS of (A,B) rhGH CRS and (C,D) heat-exposed and stored rhGH CRS using a PB-PVS coated capillary. (A,C) BPE (*m/z* 1500-3000), the asterisk indicates an unknown non-proteinaceous unknown compound. (B,D) Mass spectra obtained in the apex of the indicated peaks. Conditions: BGE, 75 ammonium formate (pH 8.5); sheath liquid, acetonitrile-water-formic acid (75/25/5, v/v/v) at 4 µL/min. Sample preparation and other conditions, see Experimental section.

could be explained by the formation of two sulfonic acid groups from a protein disulfide bridge in addition to the double methionine oxidation described above. This would explain the gain of negative charge (two sulfonate groups) and molecular mass (eight oxygen atoms) leading to the observed electrophoretic mobility and molecular weight. Disulfide bridge breakage and oxidation has been described for bovine growth hormone [39], but to our knowledge not for rhGH. Considering its molecular mass (22253.3 Da) and migration time (12.5 min), the component causing peak 4 most probably is the singly deamidated form of the sulfonated/oxidized product (peak 3). So, from the CE-TOF-MS results it can be concluded that exposure of rhGH CRS in water to mild heat followed by prolonged storage can lead to oxidation of the protein accompanied by deamidation.

3.2 Oxytocin

Oxytocin is a nonapeptide hormone drug containing an internal disulfide bridge, which is used to induce or stimulate labor and to prevent post-partum hemorrhage. It was

demonstrated that oxytocin is susceptible to pH-dependent degradation at elevated temperatures [40]. Oxytocin itself has been analyzed before with CE-UV [41] and CE-MS [42], however, CE-MS characterization of degradation products has not been reported. Here, we investigated the possibility of CE-TOF-MS to reveal the composition of heat-stressed samples of oxytocin. Oxytocin solutions of pH 2.0, 4.5, 7.0, and 9.0 that had been exposed to 70°C for 64 h were available for analysis. Oxytocin is a basic peptide (p*I* 8.7), therefore, a PB-DS-PB capillary coating was selected for CE-MS in combination with a low-pH BGE. Under these conditions, the peptide and capillary wall will be positively charged and analyte adsorption will be minimized.

Separation conditions were optimized using the heat-exposed solution of oxytocin (50 µg/mL) at pH 2.0. With a BGE of 50 mM acetic acid (pH 3.0) the BPE obtained with CE-MS revealed one main peak with a plate number of ca. 100,000. The mass spectrum of this peak comprised of distinct signals at several *m/z* values, with the most intense at *m/z* 1007.43, which agrees well with the mass of protonated oxytocin. In order to optimize the CE performance, the concentration acetic acid of the BGE (pH 3.0) was varied between 50 and 1000 mM. The plate number of oxytocin increased with increasing BGE concentration reaching an optimum (300,000) at 525 mM acetic acid. Moreover, with increasing BGE concentrations the CE resolution improved, revealing additional, partially separated, peaks. Acetic acid concentrations above 525 mM did not result in higher plate numbers or better separation. Variation of the pH (3.0-5.0) of a BGE of 525 mM acetic acid did not result in improved separation. 525 mM acetic acid (pH 3.0) then was selected as BGE for further analysis. Under these conditions, migration time RSDs for oxytocin were less than 1.4% (n=5), demonstrating the good performance of the PB-DS-PB CE-TOF-MS system.

The BPE obtained upon CE-MS analysis of oxytocin degraded at pH 2.0 showed five partially separated peaks. The mass spectrum of the main peak (8.0 min) was dominated by an intense signal at *m/z* 1007.43, whereas the mass spectra of the degradation products migrating at 6.8 min and between 7.0-7.6 min, showed most abundant signals at *m/z* 1009.39 and 1008.41, respectively. Using these masses, extracted ion electropherograms (EIEs) were constructed (Figure 6.2A). The EIE of *m/z* 1007.43 represents the unmodified oxytocin (single peak), whereas the EIE of *m/z* 1008.41 exhibits three peaks. Considering the migration times of these three degradation products with respect to oxytocin, they probably are less positively charged than oxytocin. In combination with the gain in mass of about 1 Da compared to oxytocin, one can conclude that these degradation products most likely are the result of single deamidations of oxytocin. Indeed, oxytocin contains three possible deamidation sites and apparently the three mono-deamidated oxytocins can be separated by CE. The EIE of *m/z* 1009.39 reveals the presence of bisdeamidated oxytocin (Figure 6.2A). As the peak shows an unresolved shoulder we concluded that at least two bisdeamidated variants have been formed during heat degradation.

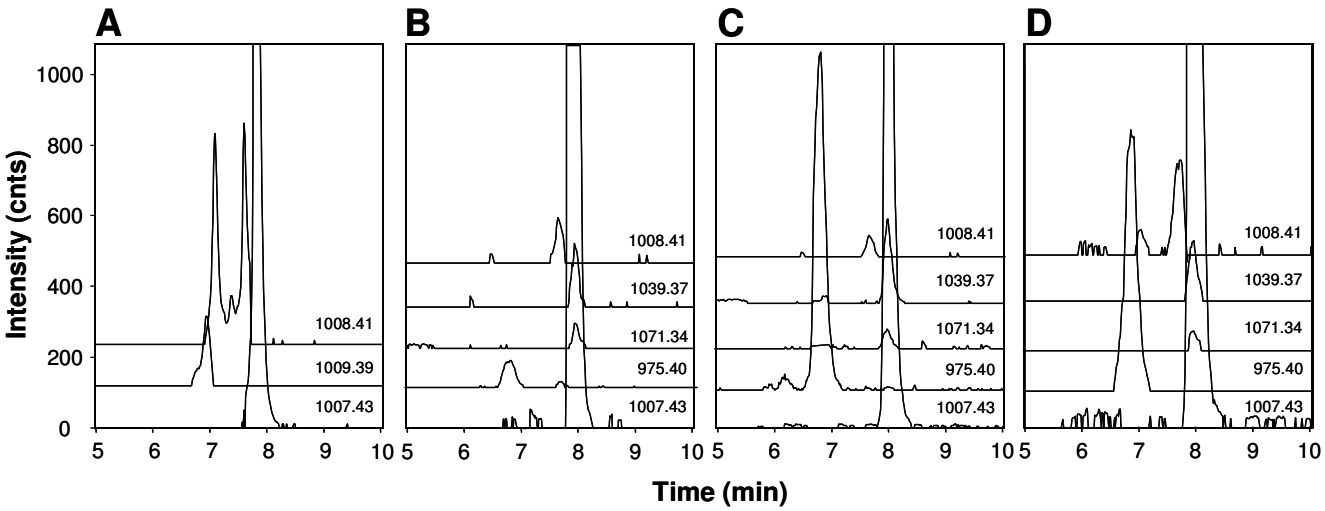


Figure 6.2. CE-TOF-MS of heat-exposed oxytocin solutions of (A) pH 2.0, (B) pH 4.5, (C) pH 7.0, and (D) pH 9.0 using a PB-DS-PB coated capillary. EIEs are at indicated *m/z* values (±0.01 Da). Conditions: BGE, 525 mM acetic acid (pH 3.0); Sheath liquid, isopropanol-water-acetic acid (75/25/0.1, v/v/v) at 2 µL/min. Sample preparation and other conditions, see Experimental section.

Oxytocin samples that were solved in phosphate buffer of pH 4.5, 7.0, and 9.0 and exposed to 70°C for 64 h were also analyzed with CE-MS, showing several degradation products (Figure 6.2B-D; Table 6.1). Clearly, deamidation is most predominant at pH 2.0; the pH 4.5-9.0 samples show only minor mono-deamidation and no bisdeamidation. Interestingly, these samples show two degradation products which comigrate with oxytocin. The mass increase of 31.95 Da and 63.91 Da with respect to oxytocin indicates that these two products most likely are the result of the addition of one and two sulfur atoms to their molecular structure. These modifications do not induce a change of molecular charge, and therefore most probably are not separated. Although unusual, trisulfide formation as a result of a β-elimination process caused by heat exposure has been reported [43]. The samples dissolved in buffer of pH 7.0 and 9.0 showed another significant degradation product with *m/z* 975.40 at a migration time of 6.8 min. From the isotope pattern it can be concluded that the compound is doubly protonated and thus has a molecular weight of 1948.8 Da. This gain in mass with respect to oxytocin suggests the product to be the result of a dimerization process involving

Table 6.1. Compounds observed with CE-TOF-MS in heat-exposed oxytocin solutions of various pH.

m/z (charge)	Compound	Relative peak area (%)			
		pH 2.0	pH 4.5	pH 7.0	pH 9.0
975.40 (2+)	Oxytocin "dimer"	N.D.[a]	2.4	14.5	9.7
1007.43 (1+)	Oxytocin	60.6	90.6	75.8	80.3
1008.41 (1+)	Deamidated oxytocin	31.4	1.4	1.1	2.8
1009.39 (1+)	Bisdeamidated oxytocin	8.0	N.D.	N.D.	N.D.
1039.37 (1+)	"Trisulfide" oxytocin	N.D.	4.8	7.0	5.7
1071.34 (1+)	"Tetrasulfide" oxytocin	N.D.	0.9	1.7	1.4

a. N.D., not detected

loss of two sulfur groups [40]. The exact mechanism of the formation of this product is unclear. In a recent study applying reversed-phase liquid chromatography (RPLC)-MS [40], the same degradation products of oxytocin were found as with our CE-TOF-MS method. The CE-MS analysis time is considerably shorter (10 min vs. 40 min), however, in contrast to CE, RPLC allowed separation of degradation products containing sulfur modifications such as tri- and tetra-sulfide bridges.

3.3 Recombinant human interferon-β-1a

The drug rhIFN-β is a 23-kDa N-glycosylated protein (Asn^{80}), which is used for treatment of multiple sclerose. Over the past years, biosimilars of rhIFN-β have been introduced onto the market. It has been demonstrated that the glycosylation of rhIFN-β may significantly affect its biological activity [44]. Therefore, establishment of the glycoform heterogeneity among batches of rhIFN-β can be of great importance. Moreover, glycoform profiling can be a tool for characterizing and comparing rhIFN-β products, including biosimilars. As protein glycosylation affects charge and size of proteins, CE – with its charge-to-mass separation mechanism – can be very useful for glycoprotein profiling. We studied whether rhIFN-β can be analyzed with our CE-TOF-MS system and the information that can be derived from the obtained data.

rhIFN-β is a basic protein (p*I* 9.6) and, therefore, to avoid protein adsorption to the capillary wall, a PB-DS-PB coated capillary was selected in combination with an acidic BGE. Previously, we found a 50 mM acetic acid BGE (pH 3.0) to be optimal for basic proteins [33]. The same BGE appeared to provide good separation of rhIFN-β glycoforms. The composition of the sheath liquid was briefly optimized. Selection of an isopropanol content of 25% and a relatively high acetic acid concentration (0.5%) appeared to be important to achieve good signals for rhIFN-β. So, a sheath liquid of isopropanol-water-acetic acid (25/75/0.5, v/v/v) at 2 μL/min was applied. Typical migration time RSDs for rhIFN-β under the selected conditions were less than 1.6% (n=5).

The BPE obtained after CE-MS of rhIFN-β (450 μg/mL) shows a distinct pattern of partially resolved bands (*i.e.* glycoforms) between 7.8 and 10.0 min (Figure 6.3A). Figure 6.3B shows the deconvoluted mass spectrum obtained in the apex of the most intense peak (9.2 min), indicating a molecular mass of 22375 Da. Some minor signals of other glycoforms with masses of 22082 and 22743 Da were also observed in the spectrum. Deconvoluted spectra of other peaks in the BPE also showed multiple compounds. For example, the deconvoluted spectrum obtained for the peak at 8.5 min shows at least five proteinaceous compounds (Figure 6.3C). In order to obtain a full picture of the number of different glycoforms, deconvoluted mass spectra were constructed with 0.1-min intervals in the range of 7.8 to 10.0 min. From these mass spectra a total of ten distinctive masses (Table 6.2) representing different glycoforms could be determined; unglycosylated rhIFN-β (20027 Da) was not detected. To assign the glycoforms, mass differences between the ten glycoforms

were determined. Merely differences of 291 Da and 365 Da were found, indicating the loss or gain of either a sialic acid unit or a hexose N-acetylhexosamine (HexHexNac) unit, respectively (Figure 6.3B and 6.3C). In addition, it has been reported that the major glycoform rhIFN-β is a fucosylated disialylated biantennary structure with four hexose and five N-acetylhexosamine units [44,45]. This glycoform, including the amino acid chain of rhIFN-β, has a molecular weight of 22375 Da. Thus, based on this glycan structure and the calculated mass differences, the most probable glycan composition of the other nine glycoforms were derived (Table 6.2). These results match well with a previous report on ESI-MS of rhIFN-β which revealed six glycoforms [44]. Interestingly, our CE-MS method allows detection of additional, low abundance glycoforms. This underlines the importance of an efficient separation prior to MS analysis in order to prevent signal suppression and allow reliable assignment.

For each assigned glycoforms an EIE was constructed using the most abundant *m/z* value of the glycoform's respective charge envelope. Four selected EIEs are depicted in Figure 6.3D. From the EIEs, relative peak areas for all ten detected glycoforms were obtained leading to a quantitative glycoform distribution, assuming an equal molar detector response per glycoforms (Table 6.2). The glycoform with the lowest abundance was almost 1% in area with respect to the most abundant isoform, but could still be detected reliably. Relative

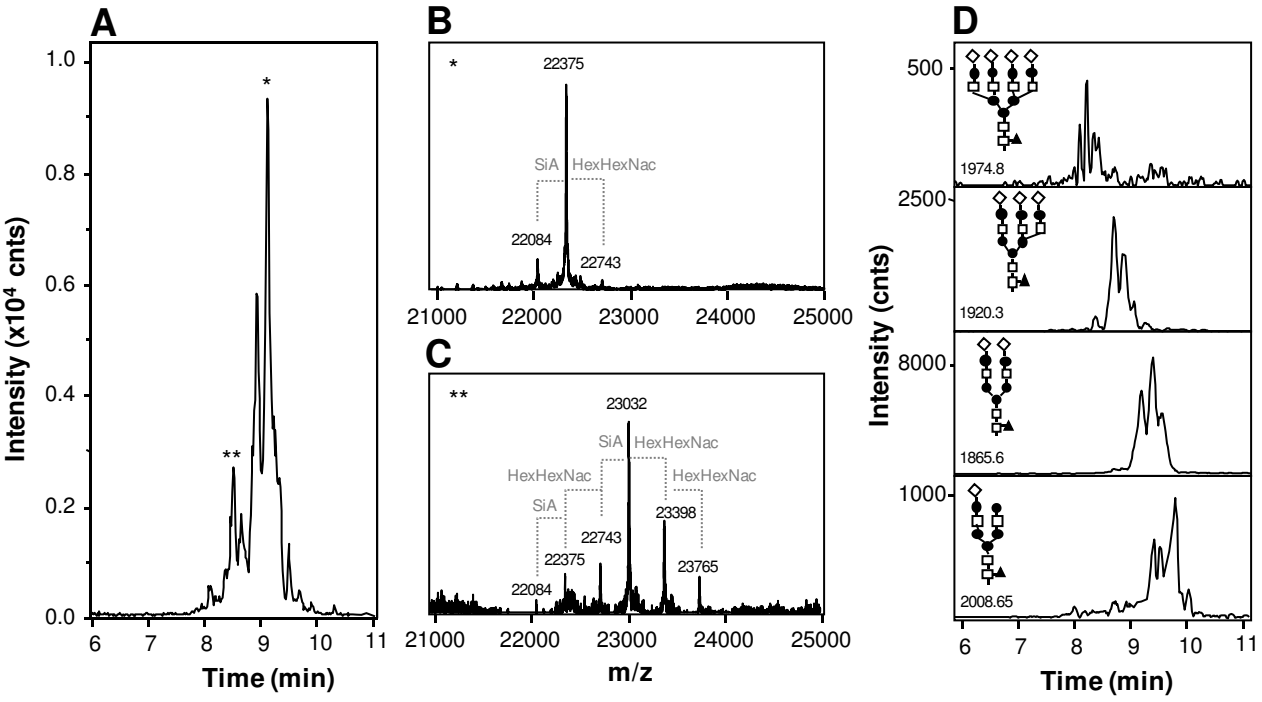
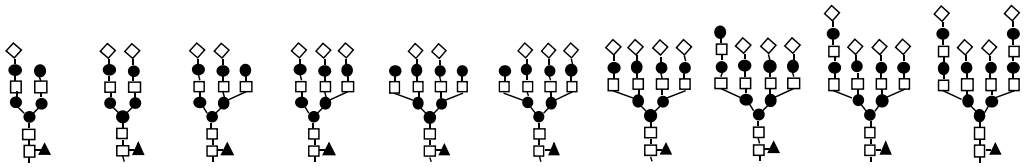


Figure 6.3. CE-TOF-MS of rhIFN-β using a PB-DS-PB coated capillary. (A) BPE (*m/z* 1500-3000). Deconvoluted mass spectrum obtained in the apex of the peak migrating (B) at 9.1 min (*) and (C) at 8.5 min (**). (D) EIEs are at indicated *m/z* values (± 0.2 Da); assigned glycan structures are shown as insets. Symbols: ●, hexose; ▲, fucose; □, N-acetylhexosamine; ◇, sialic acid. Conditions: BGE, 50 mM acetic acid (pH 3.0); Sheath liquid, isopropanol-water-acetic acid (25/75/0.5, v/v/v) at 2 μL/min. Sample preparation and other conditions, see Experimental section.

Table 6.2. Molecular mass, glycan composition, relative peak area and migration time for rhIFN-β glycoforms as observed with CE-TOF-MS.

Deconvoluted mass	22084	22375	22743	23032	23105	23398	23692	23765	24054	24424
Glycan[a]										
Relative peak area (%)[b]	6.3	52.3	6.7	12.0	5.5	9.3	1.8	4.2	1.3	0.6
Migration time (min)[c]	9.5	9.1	8.9	8.5	8.6	8.5	8.1	8.3	8.0	7.9

a. Symbols: ●, hexose; ▲, fucose; □, N-acetylhexosamine; ◇, sialic acid.

b. Total area of peaks observed in the respective EIE.

c. Migration time of the most intense peak observed in the respective EIE.

abundance of glycoforms may show useful for in-process and quality control of rhIFN-β batches or to compare biosimilar products. From the EIEs it is clear that the overall migration time of the glycoforms decreases with the degree of sialytion (Figure 6.3D). Differences in the number of HexHexNAc groups present in a glycoform with the same number of sialic acid groups only lead to a slight change of migration time (Table 6.2). These observations are in good agreement with results reported for CE-MS of other glycoproteins [12,46,47]. It should be noted that for each glycoform no well defined peak but rather a reproducible pattern of partially separated compounds was observed (Figure 6.3D). We speculate the peak clusters to be glycan isomers with slightly different electrophoretic mobilities.

4. Concluding remarks

CE-TOF-MS methods applying on noncovalently coated capillaries have been evaluated for biopharmaceutical analysis. The acidic protein rhGH could be characterized using a PB-PVS capillary coating and a medium-pH BGE, whereas the PB-DS-PB coated capillary in combination with a low-pH BGE appeared to be very suitable for the analysis of the basic pharmaceuticals oxytocin and rhIFN-β. Highly similar and/or related degradation products and glycoforms could be assigned by the combination of CE with TOF-MS. From the presented results it can be concluded that CE is especially suitable for the separation of protein modifications leading to charge differences. For example, degradation products as result of deamidation or sulfonic acid formation were efficiently resolved from the parent compound. Also protein glycoforms which differ in number of sialic acid groups could be separated. In case that modifications did not affect the overall protein charge the high mass resolution and accuracy of the TOF-MS still allowed assignment of components. So we believe there is strong potential for CE-TOF-MS systems using noncovalent coated capillaries for the purity and stability analysis of biopharmaceuticals. Moreover, the CE-MS systems

seem very useful for highly specific glycoform profiling. Currently, we are studying the usefulness of CE-TOF-MS systems for the characterization and comparison of biosimilars.

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Chapter 7

Performance of a sheathless porous tip sprayer for capillary electrophoresis – electrospray ionization-mass spectrometry of intact proteins

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Abstract

The performance of a prototype porous tip sprayer for sheathless capillary electrophoresis-mass spectrometry (CE-MS) of intact proteins was studied. Capillaries with a porous tip were inserted in a stainless steel needle filled with static conductive liquid and installed in a conventional electrospray ionization (ESI) source. Using a BGE of 100 mM acetic acid (pH 3.1) and a positively-charged polyethylenimine capillary coating, a highly reproducible and efficient separation of four model proteins (insulin, carbonic anhydrase II, ribonuclease A and lysozyme) was obtained. The protein mass spectra were of good quality allowing reliable mass determination of the proteins and some of their impurities. Sheath-liquid CE-MS using the same porous tip capillary and an isopropanol-water-acetic acid sheath liquid showed slightly lower to similar analyte responses. However, as noise levels increased with sheath-liquid CE-MS, detection limits were improved by a factor 6.5-20 with sheathless CE-MS. The analyte response in sheathless CE-MS could be enhanced by using a nanoESI source and adding 5% isopropanol to the BGE, leading to improved detection limits by 50-fold to 140-fold as compared to sheath-liquid interfacing using the same capillary – equivalent to sub-nM detection limits for three out of four proteins. Clearly, the sheathless porous tip sprayer provides high sensitivity CE-MS of intact proteins.

1. Introduction

In the fields of protein chemistry, biopharmaceutical, and biotechnology there is a growing need for sensitive and selective analytical tools for the determination of intact (*i.e.*, non-digested, non-derivatized) proteins. Capillary electrophoresis (CE) shows attractive features for the highly efficient separations of intact proteins, and its combination with mass spectrometry (MS) would provide a very selective tool for protein characterization. Since the introduction of CE-MS using electrospray ionization (ESI) and the first exploratory experiments on its applicability by Smith *et al.* [1-6], CE-ESI-MS has proven to be a strong analytical tool with a wide applicability, including determination of *e.g.* drugs, metabolites and peptides, but also intact proteins [7-11].

Coupling of CE with ESI-MS is not straightforward as both the CE and ESI processes require closed electrical circuits having a common electrode at the capillary outlet. Generally, CE-MS interfacing via an ESI source can be performed in two different ways [12]. In the most frequently applied approach, the interface uses a sheath liquid that mixes with the CE effluent as it exits the separation capillary. The sheath flow serves to establish the electrical contact with the CE background electrolyte (BGE) and facilitates ESI using a conventional ion source applying a nebulization gas. The sheath liquid allows use of standard capillaries and provides electrospray stability, making the performance virtually independent of the electroosmotic flow (EOF). However, due to the dilution of the analytes leaving the capillary by the sheath liquid, the detection sensitivity – which in ESI is predominantly concentration sensitive – is compromised. Moreover, as the sheath-liquid composition usually is quite different from the BGE, the CE performance (peak widths, migration time order) may be affected [13-15].

In the second CE-ESI-MS approach, the terminating electrical contact for the CE process is made directly to the BGE just before or after it leaves the capillary. Such a so-called sheathless interface was actually used by Olivares *et al.* [1] to perform their first on-line CE-ESI-MS experiments. Usually, the capillary tip is tapered to reduce the capillary inner diameter providing good ESI conditions. As effluent volume flow rates are low, the initial droplets formed during the electrospray process are small [16], leading to more efficient ionization (*i.e.*, nanospray). The sheathless approach also allows the ESI spray tip to be positioned closer to the MS inlet and, thereby, improving ion sampling efficiencies [16,17]. As a result, enhanced sensitivity and lower limits of detection (LODs) can be obtained.

Over time, various sheathless CE-ESI-MS interface designs have been developed. The most widely used method for establishing the terminating electrical contact is coating the outer surface of the CE capillary tip with a conductive material [18-22]. Unfortunately, lifetimes of such coatings are generally limited as they suffer from deterioration under influence of the high voltages applied. Alternatively, a wire microelectrode can be inserted into the capillary channel [23,24], however, manipulation of such microelectrodes is not easy and they can cause turbulence and bubble formation in the separation capillary reducing the

separation efficiency and electrospray stability. The CE effluent can also be brought in contact with an external electrode through a hole or a locally porous section in the capillary wall [25-27], but it is quite difficult to produce such microholes or sections in a reproducible and robust way. In order to circumvent the problem of closing the electrical circuit in sheathless CE-MS interfacing, some researchers have used separate ESI sprayer tips. The electrical contact for both the CE separation and ESI process is made at the "liquid junction" of the separation capillary and the spray tip, where the terminal electrode is placed into an electrolyte surrounding the junction [28-31]. However, the alignment of the tip is critical to maintain an efficient separation and stable electrospray. Moreover, the liquid junction may also lead to dilution of the CE effluent.

Recently, Moini has introduced a porous sheathless CE-MS interface that provides electrical contact without the need for microelectrodes or liquid junctions [32]. In this design (Figure 7.1A), the last 3-4 cm of the bare fused-silica capillary are etched with hydrofluoric acid until the section becomes conductive, producing an ~5-µm thick porous wall, which is conductive when in contact with an electrolyte. The electrical contact for both the CE and ESI is achieved by letting the porous capillary outlet protrude from a stainless steel ESI needle filled with static conductive liquid allowing electrospray formation at the capillary tip. Any bubble formation at the electrode occurs outside the separation capillary, and no dilution of the CE effluent takes place. The capillary inner diameter remains unchanged (*i.e.* no tapering) so that chances of clogging are minimized. The potential usefulness of the porous tip sprayer was demonstrated by the analysis of amino acids, peptides, protein digests and protein-metal complexes [32,33].

Based on Moini's approach, a prototype high-sensitivity porous sprayer (HSPS) sheathless interface for CE-MS was recently developed in the laboratories of Beckman Coulter. We set out to test the performance of the HSPS for the CE-MS analysis of intact proteins. There is a definite requirement for increased sensitivities in CE-MS of intact proteins. Multiple charging of proteins occurring during ESI distributes the overall signal intensity over many charge states, thereby decreasing the achievable sensitivity for intact proteins [34]. Common LODs for CE-MS of intact proteins are in the low-µM or high-nM range, although it has been demonstrated that detection of protein concentrations below 200 nM is possible with CE-MS [27,35-37].

In this study, capillaries with HSPS tips were inserted in a stainless steel ESI needle filled with static conductive liquid. The inner wall of the capillaries was covalently coated with a positively-charged polymer providing a significant EOF and avoiding protein adsorption when using low-pH BGEs. The performance of the sheathless interface was first tested using a conventional ESI source. With a mixture of model proteins, we investigated parameters such as migration time, reproducibility, detection linearity and limits of detection. Comparisons with sheath-liquid interfacing were made, paying attention to the role of organic

solvent in the sheath liquid. Finally, the optimal system was tested in conjunction with a nanoESI source.

2. Materials and methods

2.1 Chemicals

Acetic acid (99.8%), ammonium hydroxide (25%) and isopropanol were obtained from Merck (Darmstadt, Germany). Anhydrous methanol, methionine enkephalin (M_w, 573.6; p*I* 5.5), insulin (from bovine pancreas; M_w, 5733; p*I*, 5.6), carbonic anhydrase II (from bovine erythrocytes; M_w, 29025 g/mol; p*I*, 5.9), ribonuclease A (from bovine pancreas; M_w, 13680; p*I*, 9.7) and lysozyme (from chicken egg white; M_w, 14304; p*I*, 11.0) were from Sigma-Aldrich (Steinheim, Germany). Methionine enkephalin was prepared by diluting a stock solution (1 mg/mL) to a concentration of 25 µg/mL with BGE. Protein test mixtures were prepared by diluting protein stock solutions (1 mg/mL) to the appropriate concentration with deionized water. A BGE of 100 mM acetic acid (pH 3.1) was prepared by diluting 0.171 mL glacial acetic acid to 30 mL with deionized water and adjusting the pH with ammonium hydroxide.

2.2 CE system

Experiments were carried out on a P/ACE MDQ™ capillary electrophoresis instrument (Beckman Coulter, Brea, CA, USA). The separation voltage was -30 kV and the capillary temperature was 20 °C. Fused-silica capillaries (total length, 100 cm; inner diameter, 30 µm, outer diameter, 150 µm) equipped with a porous tip (length, 3-4 cm) were supplied by Beckman Coulter. The HSPS tip is under development by Beckman Coulter and is not available for commercial use. The capillaries were coated with polyethylenimine (PEI); the positively-charged PEI coating is described in US Patent 6923895 B2. During storage (overnight or longer), the capillaries were filled with methanol and the tips of the capillary were immersed in methanol. At the beginning of each day before analyses were performed, the coated capillary was conditioned by flushing the capillary at 50 psi with air (10 min), methanol (20 min), deionized water (5 min) and BGE (10 min). Before each run, the capillary was flushed for 3 min at 50 psi with fresh BGE. The sample was injected for 10 sec at 5 psi (equal to 1% of the capillary volume).

2.3 CE-MS

MS detection was performed using a micrOTOF orthogonal-accelerated time-of-flight (TOF) mass spectrometer (Bruker Daltonics, Bremen, Germany). Transfer parameters were optimized by direct infusion of an ESI tuning mix (Agilent Technologies, Waldbronn, Germany).

2.3.1 Sheathless CE-MS

Two different set-ups were used to perform sheathless CE-MS interfacing. In the first approach, the capillary with the HSPS tip was placed in a grounded coaxial CE-MS sprayer (Agilent Technologies, Waldbronn, Germany) as schematically shown in Figure 7.1B. The coaxial sprayer was positioned in a conventional ESI source comprising the standard ESI endplate and capillary cap. The stainless steel sprayer was filled with BGE via the sheath-liquid entrance to establish electrical contact with the porous tip. Neither sheath flow (static conductive liquid) nor nebulization gas were applied. The protruding distance of the porous tip was optimized by electrokinetically infusing methionine enkephalin (25 µg/mL) through the PEI coated capillary at a potential of -30 kV. Optimal dry gas flow rates (see below) were lower than the minimal flow rate (~4 L/min) recommended by Bruker Daltonics for the micrOTOF instrument. However, flow rates above the optimal flow rate resulted in distortion of the electrospray during CE-MS, and no or low protein signals were obtained. As low dry gas flow rates could result in contamination of the mass spectrometer over prolonged periods of time, the dry gas flow was increased to 5 L/min when not performing CE-MS experiments. The optimized spray conditions were as follows; dry gas temperature, 180°C; dry gas nitrogen flow, 1.0 L/min; nebulizer pressure, 0.0 bar. Electrospray in positive ionization mode was achieved using an ESI voltage of -2.3 kV.

In a later stage of the research, the porous tip capillary was placed in a grounded stainless steel needle that could be positioned by an XYZ-stage (Beckman Coulter) fitting the Bruker micrOTOF instrument (Figure 7.1C). A nanospray end plate and gas diverter were installed to allow nanoESI. The porous tip protruded the grounded needle approximately 0.5 cm and the needle was filled with BGE to establish the electrical contact. After optimization, the spray conditions were the same as with the former set-up, except for the dry gas nitrogen flow (3.0 L/min) and the ESI voltage (-2.1 kV).

2.3.2 Sheath-liquid CE-MS

For sheath-liquid interfacing the capillary with HSPS tip was withdrawn into the coaxial sprayer until the tip still protruded approximately 1 mm. A flow of 2 µL/min of isopropanol-water-acetic acid (75:25:0.1, v/v/v) was applied as sheath liquid using a 1.0 mL gas-tight syringe (Hamilton, Reno, NV, USA) on a syringe pump of Cole-Parmer (Vernon Hill, IL, USA). The optimized conditions for sheath-liquid interfacing were: dry gas temperature, 180°C; dry gas nitrogen flow, 4 L/min; nebulizer pressure, 0.4 bar; ESI voltage, -4.0 kV.

2.3.3. Data analysis

CE-MS data were analyzed using Bruker Daltonics Data Analysis software. In this study, total-ion electropherograms (TIE) and base-peak electropherograms (BPE) were constructed in the range *m/z* 1000-3000. For determination of detection linearity and LOD,

extracted-ion electropherograms (EIE) for the four model proteins were constructed from their most abundant *m/z* signals. These were *m/z* 1147.7 and 1434.1 for insulin, *m/z* 1210.4, 1262.9, 1320.3, 1383.1 and 1452.2 for carbonic anhydrase II, *m/z* 1521.2, 1711.1 and 1955.5 for ribonuclease A, and *m/z* 1431.6, 1590.34 and 1789.0 for lysozyme.

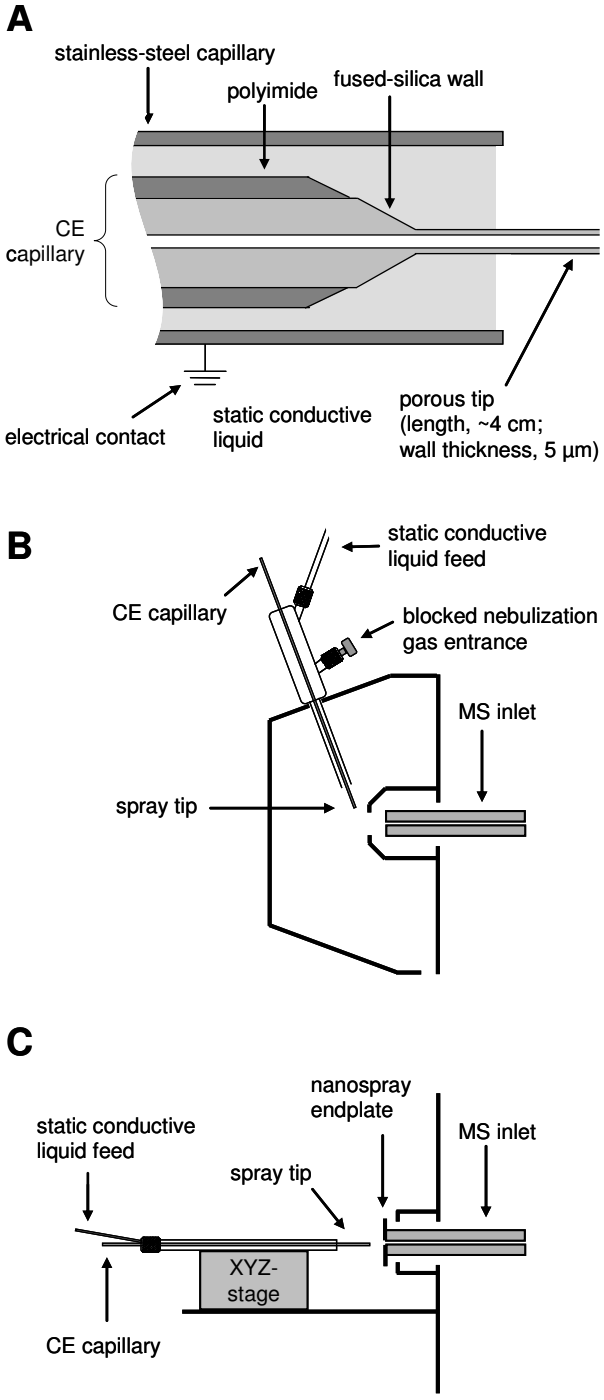

Figure 7.1. (A) Detailed representation of the HSPS capillary positioned in the stainless steel capillary. Schematic representation of the sheathless CE-MS set-up using the HSPS tip (B) in the conventional ESI source and (C) in the nanospray source.

3. Results and discussion

3.1 Sheathless CE-MS using HSPS capillaries

The capillary with HSPS tip was put into a coaxial CE-MS sprayer installed on a conventional ESI source (Figure 7.1B). The electrical contact was established by filling the stainless steel needle with BGE, which remained static as no pressure was applied and no nebulization gas was used. The contact liquid did not leave the needle (despite its tilted position) and the same liquid could be used for an entire day of measurements. As the sprayer served as the common ground for the CE and ion source, voltages for both the separation and ESI process could be independently altered. When performing sheathless CE-ESI-MS, a significant and stable EOF is of great importance to obtain good analyte signal intensities [22,38-40]. Furthermore, a typical problem in CE of proteins is that they tend to adsorb onto the fused-silica capillary wall causing band broadening and changes in EOF [41,42]. Therefore, in order to achieve stable CE-MS of intact proteins, the HSPS capillaries were coated with positively charged PEI, which induces an anodic EOF when an acidic BGE of 100 mM acetic acid (pH 3.1) is used. Under these conditions peptides and proteins exhibit a net positive charge and adsorption to the capillary wall will be avoided due to electrostatic repulsion.

To optimize the sheathless interfacing conditions, a solution of the peptide methionine enkephalin (25 µg/mL) was infused electrokinetically through the PEI-coated capillary by applying a CE voltage of -30 kV. Stable electrospray formation and an analyte signal could be established, indicating that the porous tip provided adequate electrical contact. By varying the tip protruding distance, the most optimal tip position could be determined, while simultaneously assessing the electrical contact and the stability of the electrospray process. A stable electrospray was obtained when the tip protruded the sprayer approximately 5 mm and had a distance to the MS inlet of about 10 mm, with typical electrospray currents of 130-140 nA. Subsequently, the basic performance of the PEI coating was investigated by repetitive CE-MS analysis of methionine enkephalin (25 µg/mL). In a series of 15 runs, the RSD of the EOF velocity and peptide migration time was less than 1.1%, and the RSD of the peptide peak area was less than 7%. Clearly, the PEI coating generated an EOF that provided stable separation and electrospray conditions for the 30-µm ID HSPS tip.

To study sheathless CE-HSPS ESI-MS of proteins, a mixture of insulin, carbonic anhydrase II, ribonuclease A and lysozyme was analyzed. Using a BGE of 100 mM acetic acid (pH 3.1), the four proteins were baseline separated within 10 min (Figure 7.2A). Good quality mass spectra were obtained (Figure 7.2B) allowing the assignment of the molecular masses of the respective proteins. The relatively low signal for carbonic anhydrase II in the base-peak electropherogram (BPE) can be explained by the fact that the protein signal is distributed over a considerable number of charge states. Deconvolution of the mass spectra yielded masses of 5733.6 Da (insulin), 29024.6 Da (carbonic anhydrase II), 13681.8 Da

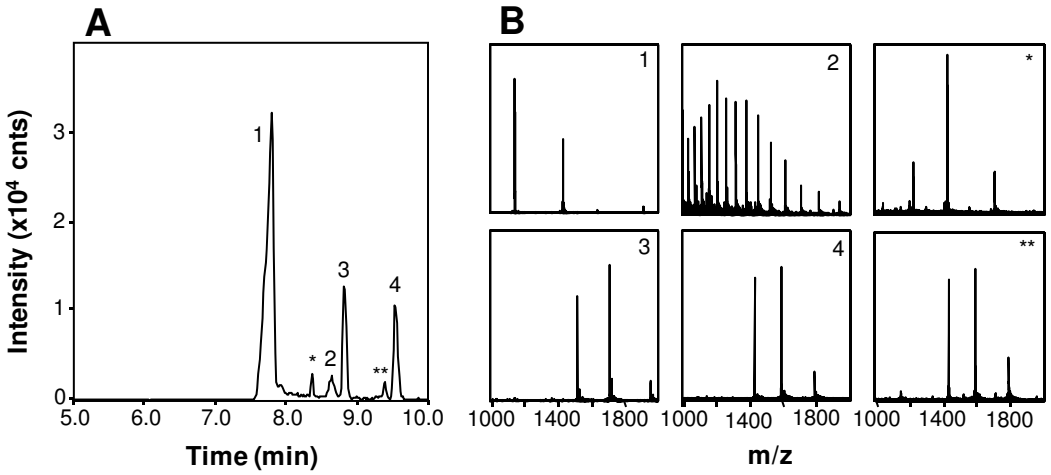

Figure 7.2. (A) BPE obtained with sheathless CE-MS of a mixture of insulin (1), carbonic anhydrase II (2), ribonuclease A (3) and lysozyme (4) (each 50 µg/mL) using the conventional ESI source. (B) Mass spectra obtained at the apices of peaks 1-4 and of peaks denoted with the asterisks. Further conditions, see Experimental Section.

(ribonuclease A) and 14304.5 Da (lysozyme), respectively, which agreed well with their expected molecular masses. The efficient CE-MS analysis also revealed the presence of two impurities (indicated by asterisks in Figure 7.2A). Considering the recorded mass spectrum (Figure 7.2B; deconvoluted mass, 8564.6 Da), the impurity migrating between insulin and carbonic anhydrase II most probably is ubiquitin. The compound migrating between ribonuclease A and lysozyme had a mass spectrum (Figure 7.2B; deconvoluted mass, 14304.3 Da) very similar to lysozyme indicating a highly-related impurity of lysozyme. Interestingly, the shorter migration time with respect to lysozyme suggests the impurity to be a modification involving a loss of postive charge. A possible explanation could be the substitution of a lysine for glutamine in the lysozyme molecule leading to a less basic protein and a mass difference of only 0.04 Da [43].

The overall performance of the sheathless CE-HSPS ESI-MS system was further evaluated by assessing the repeatability, detection linearity and limits of detection (LODs) for the test proteins. Migration time RSDs for all four proteins were less than 1%, whereas peak area RSDs were within 9% (Table 7.1). Plate numbers ranged from $0.5 \cdot 10^5$ (insulin) up to $1.5 \cdot 10^5$ (ribonuclease A). Clearly, the sheathless CE-MS system allows repeatable and efficient analyses of intact proteins. To check for linearity of signal, protein mixtures with concentrations between 0.1 and 100 µg/mL of each protein were prepared and every solution was analyzed in triplicate. Extracted ion electropherograms (EIEs) were constructed for each protein using the *m/z*-values of their most abundant signals (see Experimental section) and peak areas were determined. For all proteins, good linear relationships (Table 7.2; R^2 >0.974) between injected concentration and obtained peak areas were obtained. Table 7.2 also lists the LODs (S/N=3) achieved with sheathless CE-HSPS ESI-MS. Low-nanomolar concentrations could still be detected indicating very favorable LODs for CE-MS of intact proteins. This

Table 7.1. Repeatability of migration time and peak area (n=15) of the four model proteins (each 50 µg/mL) analyzed with sheathless CE-MS using capillaries with HSPS tips.

Protein	Migration time RSD (%)	Peak area RSD (%)
Insulin	0.63	8.5
Carbonic anhydrase II	0.61	6.3
Ribonuclease A	0.68	8.4
Lysozyme	0.74	7.0

Table 7.2. Linearity (R^2)[a] and LODs (nM)[b] for the four model proteins obtained with CE-MS with HSPS sheathless and sheath-liquid interfacing with a conventional ESI source, and HSPS sheathless interfacing with a nanoESI source.

	Sheathless – conventional ESI source[c]		Sheath liquid[c]		Sheathless – nanoESI source[d]	
Protein	R^2	LOD	R^2	LOD	R^2	LOD
Insulin	0.998	16	0.992	106	0.999	1.28
Carbonic anhydrase II	0.974	4.4	0.981	79	0.989	0.58
Ribonuclease A	0.983	2.3	0.989	33	0.992	0.62
Lysozyme	0.997	2.1	0.990	41	0.997	0.50

a. Concentration range, 0.1-100 µg/mL (sheathless conventional ESI source), 1-100 µg/mL (sheath liquid) and 0.05-25 µg/mL (sheathless nanoESI source).
b. Concentration to yield S/N ratio of 3 as calculated by extrapolation from 1-µg/mL injection. For carbonic anhydrase analyzed with sheath-liquid interfacing, a 5-µg/mL injection was used.
c. BGE, 100 mM ammonium acetate (pH 3.1).
d. BGE, 100 mM ammonium acetate (pH 3.1) containing 5% (v/v) isopropanol.

good sensitivity is primarily due to enhanced protein responses and reduced noise, as will be outlined in the next section.

3.2 Comparison with sheath-liquid CE-MS

In order to assess the actual gain in signal provided by the sheathless CE-MS set-up with the HSPS, a comparison with sheath-liquid CE-MS was made. The analyses were performed on the same days, in order to prevent day-to-day variability from influencing the results. The same capillary (that is, with the HSPS tip) was used to carry out sheath-liquid interfacing using the same BGE and injection volume. As a coaxial sprayer has been used to support the HSPS capillary, sheath-liquid interfacing conditions could be achieved simply by withdrawing the porous tip into the stainless steel needle (leaving a 1-mm protrusion) and connecting the sheath-liquid supply and nebulization gas. To study the effect of the sheath flow, BGE was first used as sheath liquid at a flow rate of 2 µL/min. CE-MS analysis of the protein test mixture under these conditions showed a dramatic decrease of analyte response as compared to sheathless CE-MS with the HSPS (Figure 7.3A). These lower protein signals can probably be explained by the dilution effect of the sheath liquid causing reduced concentrations

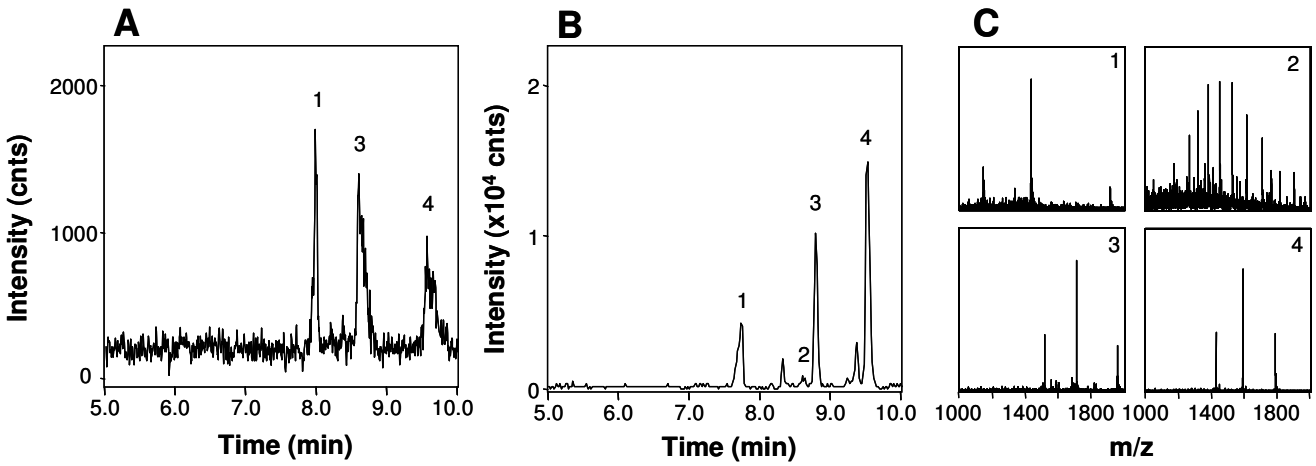

Figure 7.3. BPE obtained with sheath-liquid CE-MS of a mixture of insulin (1), carbonic anhydrase II (2), ribonuclease A (3) and lysozyme (4) (each 50 µg/mL) using the conventional ESI source and a sheath liquid of (A) 100 mM acetic acid (pH 3.1) and (B) isopropanol-water-acetic acid (75/25/0.1, v/v/v). (C) Mass spectra as obtained in the apices of peaks 1-4 in panel B. Further conditions, see Experimental Section.

of analyte in the solution that is electrosprayed. Moreover, the larger volume flow will induce formation of larger electrospray droplets, which may lead to less efficient protein ionization.

It is well known that addition of organic solvents, such as acetonitrile, methanol, and isopropanol, to the sheath liquid can significantly enhance analyte ionization [44]. In a former sheath-liquid CE-MS study of intact proteins [45], we have found a sheath liquid of isopropanol (IPA)-water-acetic acid (75/25/0.1, v/v/v) very suitable to achieve good protein signals. Applying this sheath liquid at a flow rate of 2 µL/min resulted in much improved signal intensities for the test proteins with the CE-MS system (Figure 7.3B). Furthermore, good protein separation and linear protein signals (Table 7.2) were obtained, indicating the proper functioning of the sheath-liquid interfacing. The increased ionization efficiency with respect to the aqueous sheath liquid was a direct result from the addition of IPA. Compared to water, IPA has a lower surface tension and evaporates faster. Consequently, smaller initial electrospray droplets are formed and a more efficient disintegration of the droplets occurs, resulting in more gas phase analyte ions and a higher MS signal [44]. Additionally, compared to sheathless interfacing, the introduction of the sheath liquid induced an alteration of the protein mass spectra (Figure 7.3C). A shift towards lower charge states (higher *m/z*) is observed, which is most likely caused by the higher gas-phase basicity of IPA compared to water [46]. As expected, the deconvoluted masses of the proteins did not differ from those obtained with sheathless interfacing.

The addition of IPA to the sheath liquid resulted in a significant gain in protein signal (Figure 7.3B) bringing the absolute protein signals to a level that was similar (ribonuclease A, lysozyme) or about five times lower (insulin, carbonic anhydrase II) than obtained with sheathless CE-HSPS ESI-MS (*cf.* Figure 7.3A). However, the LODs for sheath-liquid CE-MS as obtained from the EIEs of the proteins (33-106 nM), are considerably higher than for

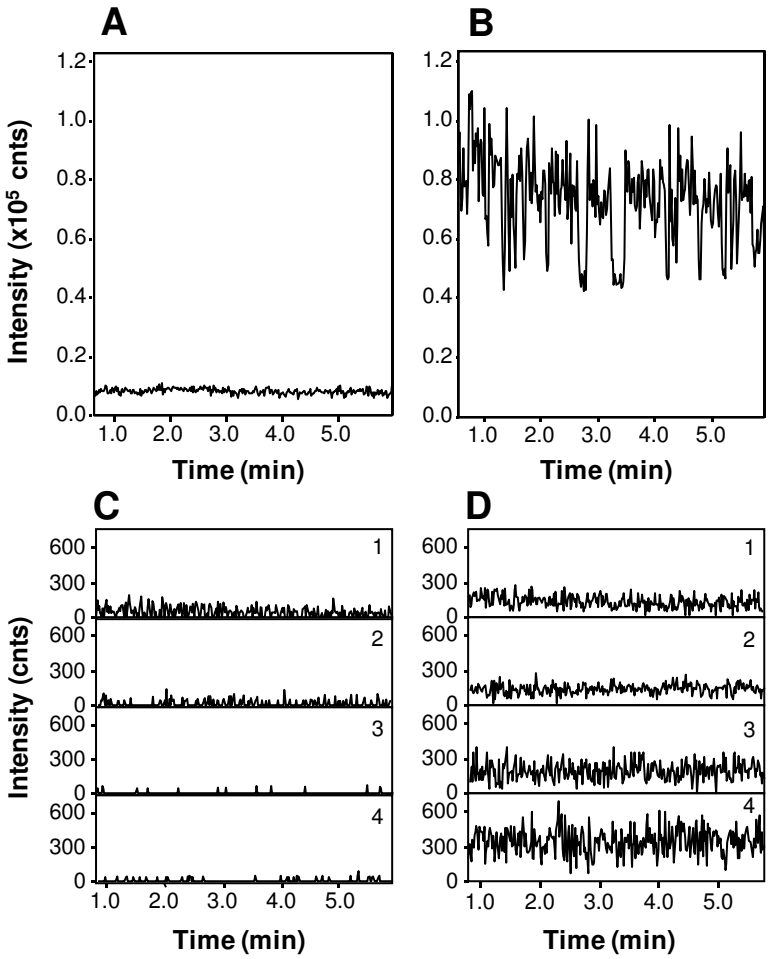

Figure 7.4. Baseline signal and noise in the 0.9-5.9 min interval during (A, C) sheathless CE-MS and (B, D) sheath-liquid CE-MS of the protein test mixture using the conventional ESI source. (A, B) Total-ion trace; (C, D) Extracted-ion traces for insulin (1; *m/z* 1434.1), carbonic anhydrase II (2; *m/z* 1262.9), ribonuclease A (3; *m/z* 1711.1) and lysozyme (4; *m/z*/ 1590.3). Further conditions, see Experimental Section.

sheathless CE-HSPS ESI-MS (Table 7.2). This can be explained by the fact that the baseline noise in sheath-liquid CE-MS is much higher, as is illustrated in Figure 7.4. This leads to lower S/N ratios and, thus, impaired LODs. Clearly, the use of sheath liquid causes a significant chemical noise, which is nicely avoided with sheathless CE-MS.

Considering the positive effect of IPA in sheath-liquid CE-MS, we checked whether addition of IPA to the BGE could be used to enhance protein MS signals in sheathless CE-MS. The effect of IPA on protein ionization was determined by infusion of protein solutions in BGE containing different concentrations of IPA. The solutions were pushed through the capillary with HSPS tip at a flow rate of 140 nL/min, similar to the velocity of the EOF in our system, and sheathless ESI was carried out. With respect to the BGE without IPA, a gain in signal was observed with increasing percentage of IPA. Most pronounced signal increase occurred between 0% and 25% IPA, with the protein signals reaching a maximum plateau between 50% and 75% IPA. Maximum gain factors reached from 2.2 (carbonic anhydrase II)

to 4.6 (ribonuclease A). Apparently, IPA also enhances the signal in sheathless CE-MS although gains are lower than in sheath-liquid CE-MS. Unfortunately, addition of IPA to the BGE at concentrations of 10% and higher caused serious broadening of the test protein peaks and loss of resolution [47,48]. A BGE with 5% IPA still gave good separation while providing a modest gain in protein signal intensities (see also below).

3.3 Sheathless CE-HSPS ESI-MS with a nanoESI source

In the final stage of this research project we came into possession of the nano-electrospray ion source for our TOF-MS instrument. This source is especially designed to handle very low flow rates typical for CE. We carried out some preliminary experiments to find out whether further gain in sensitivity could be achieved by using the sheathless HSPS capillary in combination with the nanoESI source. The porous tip capillary was placed in a grounded needle that was positioned on an XYZ-stage (Figure 7.1C). The distance of the spray tip with respect to the MS inlet was briefly optimized by electrokinetic infusion of a methionine enkephalin solution. A distance of 3 mm provided highest peptide signals. Subsequently, the protein test mixture was analyzed at different concentrations using a BGE of 100 mM acetic acid (pH 3.1) with 5% IPA (Figure 7.5A). Despite the addition of isopropanol, the protein separation was nicely maintained and the mass spectra were not affected by the IPA present in the BGE (Figure 7.5B). More importantly, for all proteins a significant increase in sensitivity was observed when compared to sheathless CE-MS using the conventional ESI source. Overall, this led to LODs which were factors of 4 to 13 lower than obtained with the standard ESI source, and even 50 to 140 times lower than for sheath-liquid CE-MS (Table 7.2). With the sheathless CE-HSPS ESI-MS using the nanoESI source,

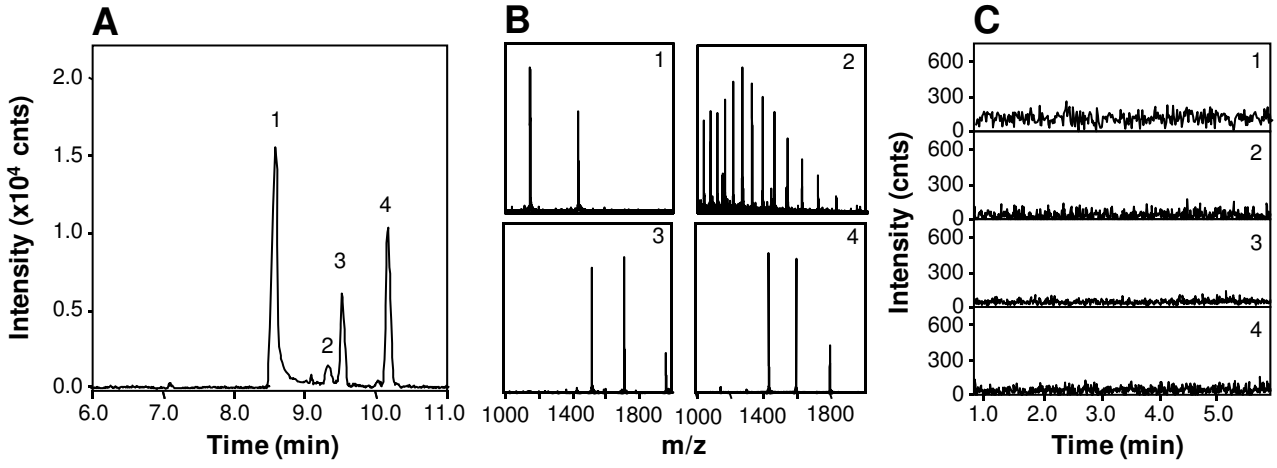

Figure 7.5. (A) BPE obtained with sheathless CE-MS of a mixture of insulin (1), carbonic anhydrase II (2), ribonuclease A (3) and lysozyme (4) (each 5 µg/mL) using the nanoESI source. (B) Mass spectra obtained in the apices of peak 1-4. (C) Baseline signal and noise of the extracted-ion traces of the test proteins (*m/z*-values used, see Figure 7.4). BGE, 100 mM acetic acid (pH 3.1) containing 5% (v/v) isopropanol. Further conditions, see Experimental Section.

sub-nM levels could even be detected for carbonic anhydrase II, ribonuclease A and lysozyme. These are highly favorable sensitivities which, to our knowledge, have not been achieved before with CE-MS for intact proteins. The sheathless CE-MS system with nanoESI source also showed good linearity (Table 7.2). A preliminary repeatability study indicated that RSDs (n=5) for protein migration time and peak area were below 0.8% and 8%, respectively. It should be noted that the baseline noise levels in the extracted-ion traces of the respective proteins were a factor 2-3 higher using nanoESI source in comparison to sheathless CE-MS with the conventional ESI source (Figure 7.5C). So, there is still room for further improvement of the system and protein detection limits in the mid-pM range might be well achievable.

4. Concluding remarks

The performance of sheathless CE-MS using a prototype HSPS for the analysis of intact proteins was studied. Stable CE and ESI conditions were established by placing the HSPS capillaries in a stainless steel needle filled with static conductive liquid. A positively-charged capillary coating in combination with a low-pH BGE provided the conditions for efficient protein separation and the EOF for effective ESI. Low-nanomolar LODs were obtained with the HSPS sheathless CE-MS system using a conventional ion source, which is a considerable improvement over sheath-liquid CE-MS employing the same capillary. This gain in performance is the result of reduced noise levels and increased analyte responses as obtained with sheathless CE-MS. The sheathless CE-HSPS ESI-MS sensitivity could be further improved by use of a nanoESI source and the addition of IPA to the BGE, achieving sub-nM LODs for three test proteins. These very favorable LODs indicate that the sheathless CE-HSPS ESI-MS can be highly useful for intact protein analysis. Considering the significant role of (reduced) noise in achieving enhanced detection limits, it might be interesting to also evaluate and compare the S/N ratios of proteins in sheathless and sheath liquid CE-MS under MS/MS conditions. The porous tip is fragile, but once the sprayer is installed in the stainless steel needle, the HSPS capillaries are easy to handle and a single capillary could be used for more than 100 runs over prolonged periods of time without any loss of performance. Beckman Coulter has developed an improved prototype containing a retractable cover on the HSPS housing to protect the porous tip when not in use. Currently, we are studying the potential of the CE-HSPS ESI-MS system for the characterization of drug-protein conjugates and the profiling of biopharmaceutical impurities, degradation products and glycoforms.

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Chapter 8

Characterization of drug-lysozyme conjugates by sheathless capillary electrophoresis – time-of-flight–mass spectrometry

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Manuscript in preparation

Abstract

Drug-protein conjugates have been widely used for the cell-specific targeting of drugs to cells that can bind and internalize the proteinaceous carrier. For renal drug targeting, lysozyme (LZM) can be used as an effective carrier that accumulates in proximal tubular cells. We used capillary electrophoresis–time-of-flight mass spectrometry (CE-TOF-MS) for the characterization of different drug-LZM conjugates. A recently developed prototype porous tip sprayer was employed for sheathless electrospray ionization (ESI) CE-MS interfacing. In order to prevent adsorption of LZM conjugates to the capillary wall, a positively-charged polyethylenimine capillary coating was used in combination with a low-pH background electrolyte. Drug-LZM products had been prepared by first coupling BOC-L-methionine hydroxysuccinimide ester (BOCmet) to lysine residues of LZM followed by conjugation with the kinase inhibitors LY364947, erlotinib, or Y27632 via a platinum(II)-based linker. CE-TOF-MS of each preparation showed good separation of the various reaction products demonstrating that drug-LZM conjugates remained stable during the CE analysis and subsequent ESI. The TOF-MS data obtained for the individual components revealed that the preparations contained LZM carrying one or two drug molecules, next to unmodified and BOCmet-modified LZM. Based on relative peak areas (assuming an equal molar response for each component) a quantitative conjugate profile could be derived for every preparation leading to apparent drug loading values of 0.42-0.59 mole drug per mole protein.

1. Introduction

By conjugating low-molecular weight drugs to a macromolecular carrier, one can override the organ distribution and cellular uptake of the coupled drug. Instead, the uptake in cells and elimination route are dictated by the pharmacokinetic profile of the carrier, and significant changes in the accumulation of the drug within tissues or cell types can be effectuated. This so-called Trojan horse approach, also referred to as advanced drug targeting, is a popular approach for improving the therapeutic index of anticancer agents, but can also be applied for other targets and diseases. The low-molecular-weight protein lysozyme (LZM; 14.3 kDa) is rapidly cleared from the blood by glomerular filtration, and has been employed as a renal-specific carrier for targeting drugs to proximal tubular cells [1]. Several approaches have been described for the linkage of drugs to LZM, which include covalent attachment via amide [2,3], ester [4] or disulfide bonds [5,6], but also coordination chemistry has been used for drug binding, *e.g.*, using a platinum linker [7-9]. Reaction conditions can often be chosen such that the average coupling ratio of drug to LZM carrier is approximately 1:1, which is preferred over extensive modification of the carrier protein.

Key issues in drug-LZM conjugate synthesis are the development of reproducible methods for preparation – to ensure batch-to-batch consistency – and the availability of appropriate characterization methodologies. Typically, characteristics of drug-protein conjugates are derived from assays that determine properties of the bulk preparation. For example, liquid chromatographic (LC) and spectrophotometric assays can be used to determine the total drug content (after release from the conjugate) and total protein content, respectively [7-9] leading to an overall drug-protein ratio (i.e. loading value). However, drug-protein conjugates usually consist of a mixture of products comprising protein molecules carrying one or more drug molecules, but also unreacted protein. So, an average drug-protein ratio alone might not be a fully appropriate indicator for the quality of conjugate preparations. More thorough compositional characterization is needed to reveal the specific conjugate stochiometry and the actual species present in the synthesis mixture. Techniques like gel electrophoresis (GE) [10,11] and matrix-assisted laser-desorption ionization – mass spectrometry (MALDI-MS) [12-14] have been used for the analysis of drug-protein conjugates. However, the molecular size-based selectivity of GE might not be adequate to separate highly-related compounds. Using direct MALDI-MS analysis of drug-protein conjugate mixtures, all components have to be ionized simultaneously and minor products might not be detected due to ionization suppression. A methodology that provides efficient separation prior to selective detection would be highly useful for characterization of drug-protein products.

Capillary electrophoresis (CE) – with its charge-to-size separation mechanism – in principle is a suitable technique to resolve the constituents of a drug-protein conjugate mixture [15]. Drug-protein conjugation often involves binding to lysine residues, which will affect the protein's molecular charge and mass, resulting in a change of electrophoretic

mobility. As CE has the intrinsic capacity to produce narrow peaks, it shows good potential for the separation of several protein modifications in a single run. Detection with high mass accuracy and resolution, such as provided by time-of-flight (TOF) -MS instruments, can yield accurate information on the molecular weight of separated proteins species. CE-MS using electrospray ionization (ESI) has proven to be a valuable tool for the characterization of proteins [16,17], including highly related species, such as glycoforms [18], biopharmaceutical degradation products [19], and biomarkers [20]. So far, the feasibility of CE-MS for the analysis of drug-protein conjugates has been indicated only by Bruins *et al.* [15]. In their study, the identity of reaction products resulting from the covalent conjugation of naproxen to LZM could be confirmed using a custom-built sheath-liquid CE-MS system. However, the resolution of the applied mass spectrometer was rather limited and drug-protein conjugate concentrations as high as 1 mg/mL had to be injected. Moreover, protein adsorption to the capillary wall resulted in peak tailing

Recently, we have demonstrated the usefulness of sheathless CE-TOF-MS for the efficient and repeatable analysis of intact proteins, including LZM [21]. The adsorption of proteins to the capillary wall was prevented by using a positively-charged capillary coating of polyethylenimine in combination with a low-pH background electrolyte (BGE). A novel prototype sheathless interface was used to couple CE with ESI-TOF-MS, allowing detection of intact proteins at the sub-nM level. The interface was recently developed in the laboratories of Beckman Coulter, based on a design introduced by Moini [22]. In this design, the 3-4 cm of the terminating end of the fused-silica separation capillary is etched with hydrofluoric acid, producing a ~5-µm thick porous capillary wall allowing electrical contact when immersed in BGE. The porous capillary outlet protrudes from a stainless steel ESI needle filled with static conductive liquid providing the means to close the electrical circuits needed for CE separation and electrospray formation at the capillary tip. In this interfacing approach, the use of sheath liquid is avoided leading to significantly improved sensitivities [21].

In the present study, the sheathless CE-TOF-MS system employing the porous tip sprayer was used to characterize preparations of drug-LZM conjugates. Kinase inhibiting drugs LY364947, erlotinib and Y27632 had been coordinately bound to LZM using a platinum-based linker (Universal Linkage System; ULS™). The composition of the resulting preparations was assessed with CE-TOF-MS using experimental conditions as previously determined for intact protein analysis [21]. Drug-protein stochiometries and conjugate profiles were derived and drug loading values were determined.

2. Materials and methods

2.1 Chemicals

Isopropanol, 99.8% (v/v) acetic acid, and 25% (v/v) ammonium hydroxide were obtained from Merck (Darmstadt, Germany). Anhydrous methanol was from Sigma-Aldrich

(Steinheim, Germany). A BGE of 100 mM acetic acid (pH 3.1) containing 5% (v/v) was prepared by diluting 0.171 mL glacial acetic acid to 30 mL with deionized water/isopropanol (95/5, v/v) and adjusting the pH with 1% (v/v) ammonium hydroxide.

2.2 Drug-lysozyme preparations

The investigated drug-LZM conjugates were synthesized as described earlier [7,8] and schematically depicted in Figure 8.1A-B. Briefly, methionine residues were attached to lysine residues of LZM by adding a three times molar excess of BOC-L-methionine hydroxysuccinimide ester (BOCmet-NHS; 15461; Fluka, Buchs, Switserland) to hen egg white LZM (62971; Fluka, Buchs, Switserland) in PBS. The product was dialyzed against water and then lyophilized. The kinase inhibitors LY364947, erlotinib, and Y27632 (Figure 8.1C-E) were coupled to a platinum-based linker cis-Pt(ethylenediamine)nitrate-chloride (ULS™). LY364947 was incubated with an equimolar amount of ULS at 37 °C for 4 hours, erlotinib was reacted overnight at 65 °C with a 3 times excess of ULS, whereas Y27632 was reacted with a 2.2 times molar excess of ULS and incubated for 16 hours at 50 °C. Conjugation of LY364947-ULS, erlotinib-ULS, and Y27632-ULS to LZM was performed by the overnight reaction of a three times molar excess of the individual kinase inhibitor-ULS complex with BOCmet-modified LZM in 0.02 M tricine/sodium nitrate buffer (pH 8.5) at 37 °C. The resulting drug-LZM conjugates (Figure 8.1B) were three times dialyzed against water, lyophilized and stored at -20 °C. Prior to CE-MS analysis, the preparations were dissolved in deionized water to a final overall protein concentration of 100 nM.

2.3 CE system

Experiments were carried out on a P/ACE MDQ™ capillary electrophoresis instrument (Beckman Coulter, Brea, CA, USA). The separation voltage was -30 kV and the capillary temperature was 20 °C. Fused-silica capillaries (total length, 100 cm; inner diameter, 30 µm, outer diameter, 150 µm) equipped with a porous tip (length, 3-4 cm) were supplied by Beckman Coulter. The high sensitivity porous sprayer (HSPS) tip is under development by Beckman Coulter and is not available for commercial use. The capillaries were coated with polyethylenimine (PEI); the positively-charged PEI coating is described in US Patent 6923895 B2. During storage (overnight or longer), the capillaries were filled with methanol and the tips of the capillary were immersed in methanol. At the beginning of each day before analyses were performed, the coated capillary was conditioned by flushing the capillary at 50 psi with air (10 min), methanol (20 min), deionized water (5 min) and BGE (10 min). Before each run, the capillary was flushed for 3 min at 50 psi with fresh BGE. The sample was injected for 10 sec at 5 psi (equal to 1% of the capillary volume).

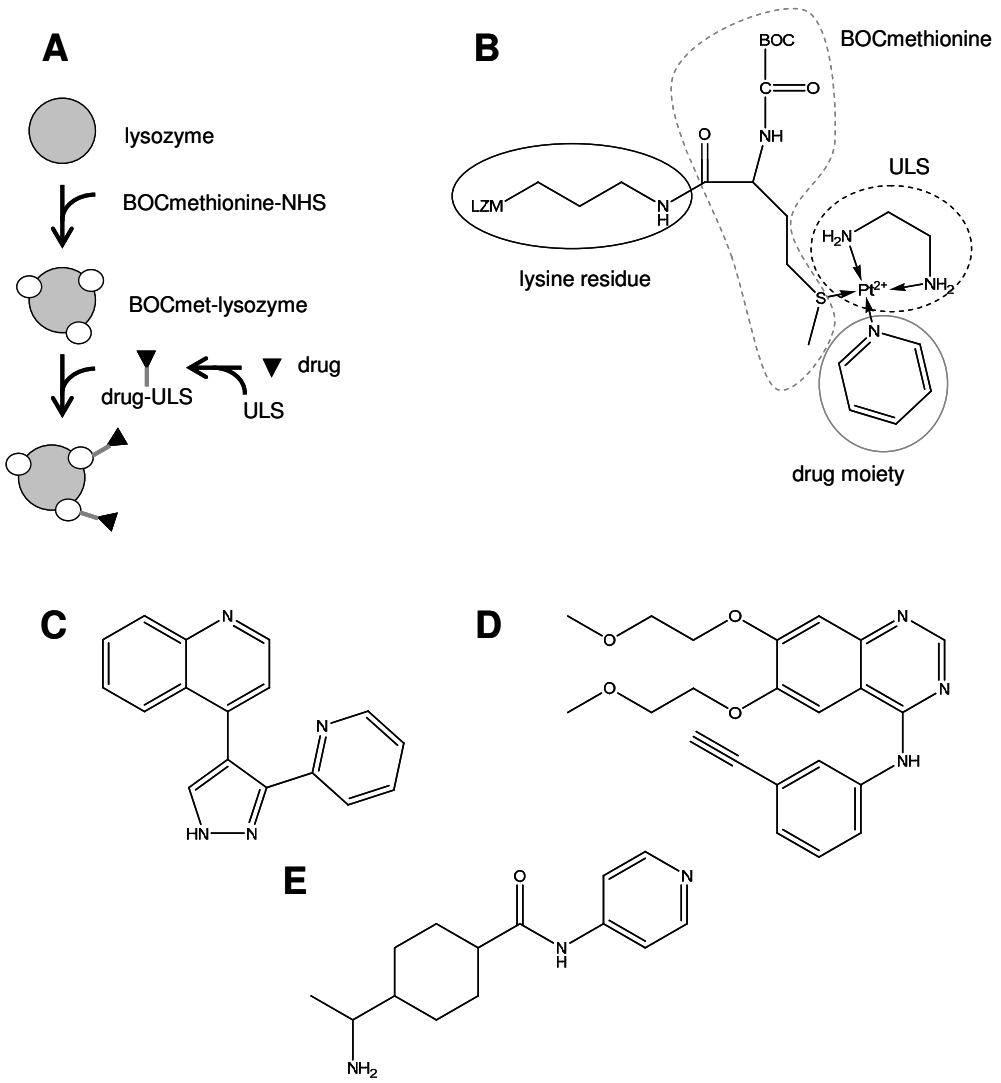

Figure 8.1. (A) Synthesis scheme of the drug-LZM conjugates. (B) Schematic representation of a drug-ULS-BOCmet-LZM conjugate. (C-E) Molecular structures of LY364947 (C), erlotinib (D), and Y27632 (E).

2.4 MS detection

MS detection was performed using a micrOTOF orthogonal-accelerated time-of-flight (TOF) mass spectrometer (Bruker Daltonics, Bremen, Germany). Transfer parameters were optimized by direct infusion of an ESI tuning mix (Agilent Technologies, Waldbronn, Germany). The capillary with the HSPS tip was placed in a grounded stainless-steel needle positioned in the ESI source of the mass spectrometer. The needle was filled with BGE (static conductive liquid) to establish electrical contact with the porous tip. The dry gas temperature and flow was 180 °C and 1.0 L/min, respectively. Electrospray in positive ionization mode was achieved using an ESI voltage of -2.3 kV. CE-MS data were analyzed using Bruker Daltonics Data Analysis software. Protein ion charge assignment and molecular weight determinations were performed using the 'Charge Deconvolution' utility of the DataAnalysis software.

3. Results and discussion

3.1 CE-MS of BOC-methionine-modified lysozyme

In order to allow conjugation of the kinase inhibitor-ULS complexes to surface exposed methionine groups of LZM, the protein was first treated with BOCmet-NHS to introduce sulfur groups for platinum coordination (Figure 8.1A). The reaction product (total protein concentration, 100 nM) was analyzed with the sheathless CE-TOF-MS system. LZM is a basic protein (pI, ~11) that readily adsorbs to fused-silica surfaces. Therefore, a positively-charged PEI capillary coating was used to induce electrostatic repulsion between the net positively-charged protein and the capillary wall, and thus effectively prevent adsorption. A BGE of 100 mM acetic acid (pH 3.1) containing 5% (v/v) isopropanol was selected as it previously had shown to be appropriate for LZM analysis while providing favorable protein MS signal intensities [21]. The base peak electropherogram (BPE) of the BOCmet-modified preparation obtained with CE-MS showed a cluster of four peaks (Figure 8.2A). Deconvolution of the mass spectra recorded in the apices of the peaks 1 to 4 resulted in molecular weights of 14994.3, 14766.1, 14535.1, and 14304.9 Da, respectively, for the detected protein species. Considering the molecular masses of LZM and the BOCmet residue

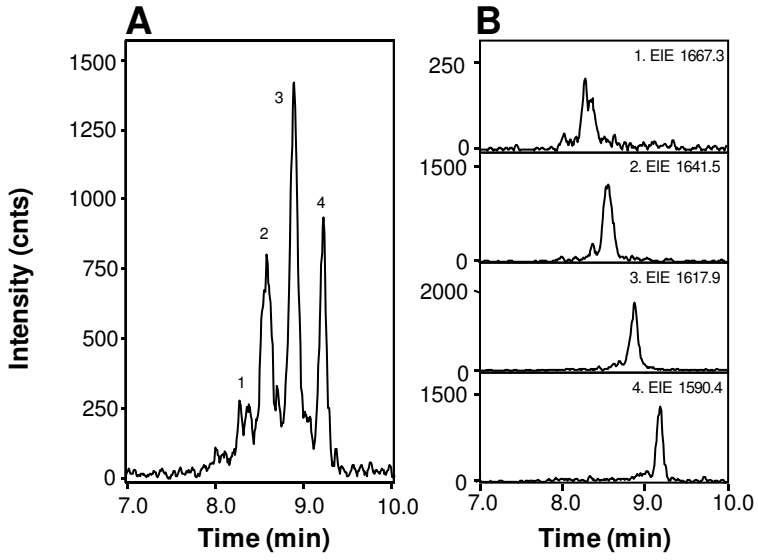

Figure 8.2. CE-TOF-MS of the BOCmet-LZM preparation. (A) BPE (*m/z* 1500-1800); (B) EIEs for the main ion of peak 1-4 constructed at the indicated *m/z* values (±0.2 Da). Peak assignment, see Table 8.1.

Table 8.1. Deconvoluted molecular masses (M_w), relative peak areas and assignment of components detected during CE-TOF-MS of the BOCmet-LZM preparation.

Peak no.	Measured M_w (Da)	Peak area (%)	Assignment
1	14994.3	5.9	$BOCmet_3$-LZM
2	14766.1	28.9	$BOCmet_2$-LZM
3	14535.1	37.4	$BOCmet_1$-LZM
4	14304.9	27.8	LZM

which is conjugated with the protein lysines, the components could be assigned to LZM (peak 4) and LZM modified with one, two or three BOCmet residues (peaks 3, 2 and 1, respectively; Figure 8.2A). Conjugation to lysine leads to a net reduction of positive charge of the protein and, thus, to a reduction of its effective electrophoretic mobility. Indeed, the migration times of the BOCmet-LZM conjugates decrease with increasing degree of conjugation. For all observed conjugates *m/z* $(M+9H)^{9+}$ was the most intense protein charge state observed in the mass spectra. This is in line with results obtained during CE-MS of acetylated LZM [23-25] and indicates that BOCmet conjugation did not cause unfolding of the protein. Using the observed signal for *m/z* $(M+9H)^{9+}$, extracted-ion electropherograms (EIEs) were constructed for the LZM conjugates (Figure 8.2B). These EIEs underline the adequate CE separation of the LZM conjugates. From the EIEs, relative peak areas were determined for all components detected in the BOCmet-LZM preparation (Table 8.1). From these results it can be concluded that under the applied conditions, $BOCmet_1$-LZM is the main reaction product. However, significant amounts of unmodified LZM are still present in the product, whereas also $BOCmet_2$-LZM and – to a much lesser extent – $BOCmet_3$-LZM has been formed.

3.2 CE-MS of drug-lysozyme preparations

Three different drug-ULS-BOCmet-LZM conjugates were synthesized based on the BOCmet-LZM preparation analyzed in Section 3.1. Drug-ULS complexes were individually added in excess to BOCmet-modified LZM, allowing the coordinate binding of the LZM-bound sulfur groups with the platinum linker (Figure 8.1). The conjugated drugs were the kinase inhibitors LY364947, erlotinib and Y27632. The reaction products (total protein concentration per preparation, 100 nM) were analyzed by CE-TOF-MS (Figures 8.3-8.5). The BPEs of the three preparations showed a pattern of peaks with migration times between 8 and 10 min (Figures 8.3-8.5A). The mass spectra recorded in the apices of the respective peaks were deconvoluted, revealing the molecular weights of the components of each drug-LZM conjugate preparation (Table 8.2). For all detected components, EIEs were constructed using the signal for the most intense protein charge state of the respective protein species (*i.e. m/z* $(M+9H)^{9+}$), showing distinct and well-resolved peaks (Figures 8.3-8.5B). Taking into account the molecular masses of the ULS linker, the kinase inhibitors, the BOCmet residue and LZM, all detected components could be assigned based on their measured molecular mass (Table 8.2). From these results it is evident that the desired drug-LZM conjugates are successfully formed using the ULS linker. Most of the produced drug conjugates had a 1:1 drug-LZM ratio, but in all preparations conjugates comprising two kinase inhibitor molecules were observed as well. It is also clear that unmodified BOCmet-LZM conjugates are still present in the preparations, indicating that under the applied conditions the ULS linking reaction does not go to completion. As could be expected, all drug-LZM preparations also contained unmodified LZM, which is part of the BOCmet-LZM preparation (see Section 3.1), and

cannot bind to the ULS linker. It is important to note that LZM nor (ULS-)BOCmet-LZM conjugates were detected at the migration times of the drug-LZM products (Figures 8.3-8.5B). This means that the platinum-coordinated drug-LZM conjugates do not dissociate during ESI. Furthermore, for all components discrete symmetrical peaks are obtained, which indicates that the conjugates are stable during CE separation. From these observations we conclude that the CE-TOF-MS results nicely reflect the actual qualitative composition of the drug-LZM conjugate preparations.

Taking the EIEs of Figures 8.3-8.5B and the peak assignments into consideration, one can see that the drug-LZM conjugates are shifted towards longer migration times with respect to their unmodified BOCmet-LZM counterparts. This can be explained by the fact that the introduction of the drug-ULS complex – which contains Pt^{2+} – to the protein goes along with an increase of the net positive charge of the overall LZM conjugate. As a consequence, the effective electrophoretic mobility of the conjugate will increase. This effect is even more evident for the Y27632-LZM conjugate (Figure 8.5) which has a significantly longer migration time than the other two kinase inhibitor-LZM conjugates. Y27632 comprises a strongly basic primary amine group which further adds to the overall positive charge of the conjugate. These observations once again underline the usefulness of CE for the characterization of drug-protein preparations.

From the EIEs constructed at *m/z* $(M+9H)^{9+}$ of the drug-LZM preparations, peak areas were determined for all detected components. Assuming an equal molar detector response for each component, a quantitative estimation of the relative composition of the preparations could be made (Table 8.2). Peak areas obtained from EIEs based on the $(M+8H)^{8+}$ or $(M+10H)^{10+}$ charge states led to virtually the same relative compositions, indicating that the protein charge state distributions are the same among the respective conjugates. As the drug-

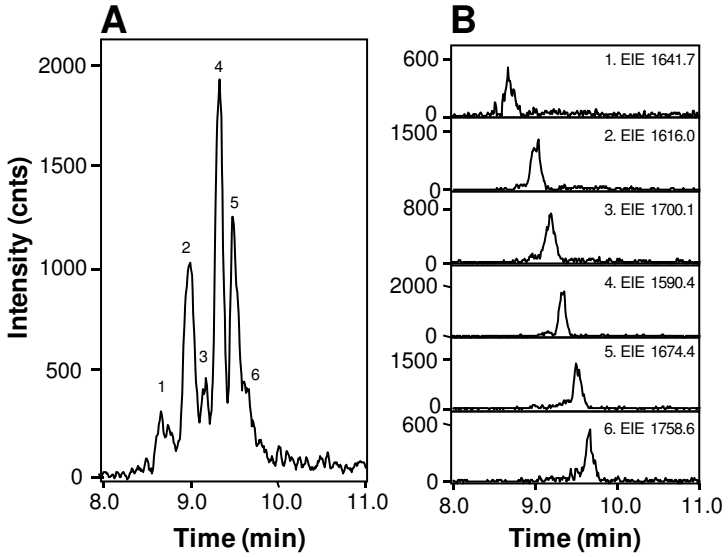

Figure 8.3. CE-TOF-MS of the LY364947-ULS-BOCmet-LZM preparation. (A) BPE (m/z 1500-1800); (B) EIEs for the main ion of peak 1-6 constructed at the indicated m/z values (±0.2 Da). Peak assignment, see Table 8.2.

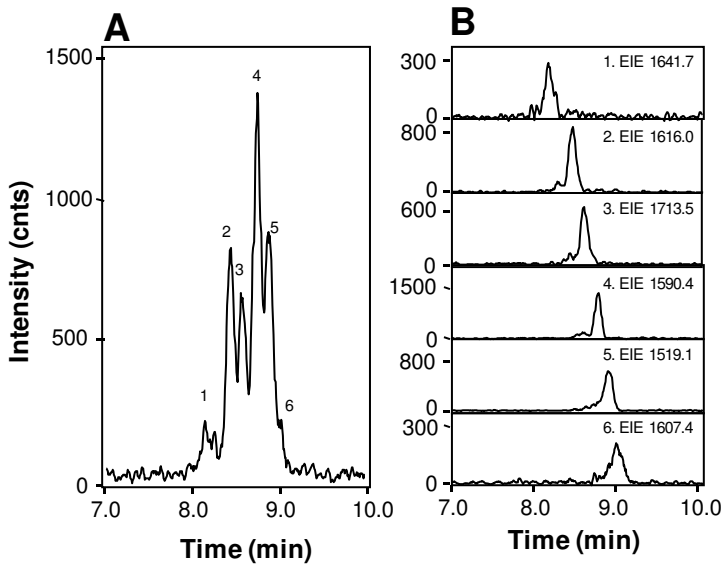

Figure 8.4. CE-TOF-MS of the erlotinib-ULS-BOCmet-LZM preparation. (A) BPE (*m/z* 1500-1800); (B) EIEs for the main ion of peak 1-6 constructed at the indicated *m/z* values (±0.2 Da). Peak assignment, see Table 8.2.

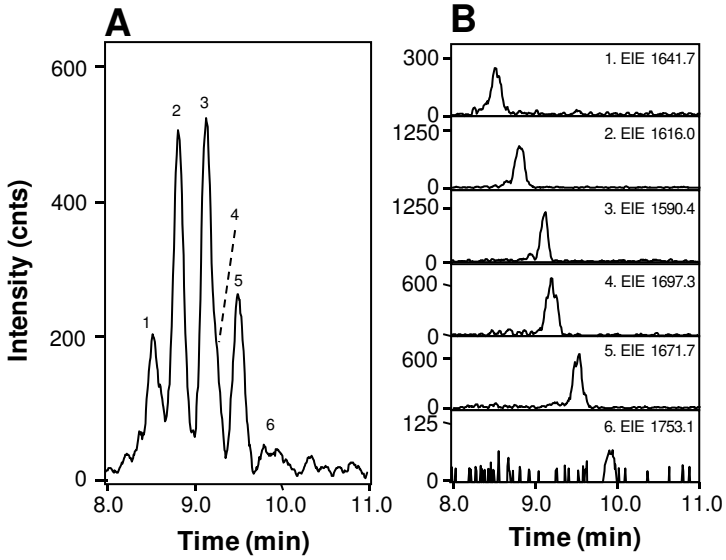

Figure 8.5. CE-TOF-MS of the Y27632-ULS-BOCmet-LZM preparation. (A) BPE (*m/z* 1500-1800); (B) EIEs for the main ion of peak 1-6 constructed at the indicated *m/z* values (±0.2 Da). Peak assignment, see Table 8.2.

LZM preparations are based on the same BOCmet-LZM product and unmodified LZM does not bind to the ULS linker, the relative LZM content of the preparations should be the same. The measured relative abundance of LZM in the preparations indeed is very similar (26-29%) and corresponds well with the relative LZM amount found in the BOCmet-LZM preparation (27.8%; Table 8.1). So, the relative peak areas appear to represent the composition in an adequate way. The results in Table 8.2 further show that 36-43% of the drug-LZM preparations consist of conjugates comprising one kinase inhibitor molecule, whereas conjugates with two drug molecules make up 2-9% of the preparations. Interestingly, a relative content as low as 2.6% – which corresponds to an injected concentration of 2.6 nM – could still be determined reliably for (Y27632-ULS)$_2$-(BOCmet)$_2$-LZM, illustrating the ability

Table 8.2. Deconvoluted molecular masses (M_w), relative peak areas and assignment of components detected during CE-TOF-MS of the three kinase inhibitor-ULS-BOCmet-LZM preparations.

LY364947			Erlotinib			Y27632)			
Peak no.	Measured M_w (Da)	Peak area (%)	Peak no.	Measured M_w (Da)	Peak area (%)	Peak no.	Measured M_w (Da)	Peak area (%)	Assignment
1	14766.6	5.9	1	14766.7	5.0	1	14766.2	10.7	$BOCmet_2$-LZM
2	14535.8	20.8	2	14535.2	18.2	2	14535.0	21.7	$BOCmet_1$-LZM
4	14305.0	28.8	4	14304.3	26.2	3	14304.5	28.2	LZM
5	15059.6	23.2	5	15179.3	28.5	5	15033.7	20.2	$(drug\text{-}ULS)_1$-$BOCmet_1$-LZM
3	15289.4	13.9	3	15409.8	13.8	4	15264.2	16.6	$(drug\text{-}ULS)_1$-$BOCmet_2$-LZM
6	15813.8	7.3	6	16055.5	8.2	6	15764.5	2.6	$(drug\text{-}ULS)_2$-$BOCmet_2$-LZM

of the sheathless CE-TOF-MS system to detect low-abundant conjugates. Taking the presence of unmodified LZM and BOCmet-LZM into account, the relative abundances reported in Table 8.2 lead to overall drug loading values (*i.e.* mole drug per mole LZM) of 0.52, 0.59 and 0.42 for the LY364947, erlotinib and Y27632 LZM preparations, respectively. Notably, combination of the results of an LC-based assay (drug content) and spectrophotometric assay (protein content) of the same preparations yielded overall loading values of 0.74, 0.87 and 0.64, respectively. Currently, we are studying the potential causes for this discrepancy of loading values. One possible reason could be that the ESI efficiency of LZM is affected by the drug conjugation, leading to underestimation of the drug-LZM conjugates with respect to LZM.

4. Concluding remarks

In this work, drug-LZM conjugates were characterized by CE-TOF-MS. A positively-charged capillary coating in combination with a low-pH BGE provided the conditions for efficient analysis basic protein species avoiding capillary-wall adsorption and peak tailing. Furthermore, sheathless CE-MS interfacing allowed detection of the conjugates in the nM-range. Due to protein charge changes induced by drug conjugation, efficient CE separation of the highly-related conjugate products could be obtained. The high mass accuracy of TOF-MS detection permitted reliable assignment of separated compounds. With the presented CE-TOF-MS method, not only drug loading values can be derived, but also insight into the stochiometry of the drug-protein binding is obtained. All these aspects embody a substantial improvement in terms of separation efficiency, sensitivity and assignment accuracy when compared to an earlier reported CE-MS method for naproxen-LZM conjugates [15]. Overall it can be concluded that CE-TOF-MS is a tool with high potential for characterization of protein-drug conjugates. Currently, we are exploring its additional possibilities for the study of albumin and antibody-based drug. A further challenging step in this field is the characterization and distinction of topological isomers. This would involve the assignment of the actual positions of conjugation on the protein backbone. For this purpose, it would be interesting to evaluate the combination of CE with electron-transfer dissociation TOF-MS, which has recently become commercially available.

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Chapter 9

Conclusions and perspectives

In this thesis capillary electrophoresis–time-of-flight mass spectrometry (CE-TOF-MS) systems using coated capillaries have been developed and studied, and their utility for the characterization of biopharmaceuticals has been evaluated and demonstrated. This final chapter provides comments and conclusions on several general and practical aspects related to the used capillary coatings, MS detection of proteins, CE-MS interfacing, and applicability of the developed methods. Furthermore, some perspectives and recommendations for further research will be given.

1. Capillary coatings

The potential of positively-charged coated capillaries for the reproducible and efficient CE analysis of intact basic proteins was evaluated using UV as well as TOF-MS detection. The preparation of the studied coatings Polybrene-dextran sulfate-Polybrene (PB-DS-PB) and polyethelylenimine (PEI) is fast and straightforward, and basically only requires rinsing of the capillary with solutions of the respective polymers. As no time-consuming and laborious procedures are needed, these coated capillaries can be rapidly prepared with good inter-capillary reproducibility. Typical CE migration-time reproducibilities for proteins obtained with the polymer-coated capillaries are within 2% RSD, even for prolonged time of use. The charged coatings studied produce a rather strong and constant electroosmotic flow (EOF). The EOF induced by the PB-DS-PB coating is virtually independent of the pH of the background electrolyte (BGE). Such a strong EOF may be somewhat less favorable when high protein resolution is pursued with CE, and, therefore, high plate numbers are needed to achieve sufficient separation in a relatively short time. Fortunately, the PB-DS-PB and PEI coating – and also the Polybrene-poly(vinylsulfonic acid) (PB-PVS) coating used for acidic proteins – effectively prevent protein adsorption to the capillary surface and, consequently, facilitate efficient protein separations by CE providing narrow peaks. For the PB-DS-PB coating, potential protein adsorption was investigated in-situ using the surface-specific detection technique evanescent-wave cavity ring-down spectroscopy (EW-CRDS). This technique confirmed the superior effectiveness of the PB-DS-PB coating in minimizing adsorption of basic proteins. The triple layer coating showed significantly less reversible and irreversible protein adsorption when compared to a PB single layer coating.

2. MS detection

As is amply demonstrated in this thesis, the use of electrospray ionization mass spectrometry (ESI-MS) as a selective detection technique in CE provides highly useful information on the molecular weight of separated intact proteins. It is important to note that the capillary coatings needed for efficient protein separation showed full compatibility with ESI-MS detection. No signals related to coating agents were detected during CE-MS analyses of proteins, and the coatings also did not cause any protein ionization suppression. Furthermore, the significant EOF generated by the charged coatings was found to be

advantageous in order to achieve adequate and reproducible ESI interfacing conditions. The use of volatile BGEs is indicated in CE-ESI-MS of proteins. Still, BGE concentrations should be kept as low as possible in order to attain good sensitivities. Especially, BGEs containing ammonium ions may cause significant ionization suppression of intact proteins in ESI and potentially can impede the analysis of low abundance degradation products or impurities.

TOF-MS provides the high mass resolution and high accuracy required to confidently assign mass differences among protein species even if the compounds are not fully separated by CE. For example, the partial CE separation of glycoforms of interferon-β was fully compensated by the detection of accurate mass by TOF-MS, allowing assignment of the various protein isoforms present in the sample. Nevertheless, reliable assignment of protein degradation products or impurities that have very small mass differences with respect to the parent protein (*e.g.* as result of deamidation) requires full CE separation in order to avoid disturbing overlap of the compounds' isotope patterns in the measured mass spectra. Efficient CE separation prior to TOF-MS detection might also be essential to assure that the detected mass belongs to an actual sample component and is not formed during ESI of the analyzed protein.

3. CE-MS interfacing

Sheath-liquid interfacing was used for a significant part of the CE-MS studies described in this thesis. The purpose of the sheath liquid is to establish electrical contact with the CE effluent and to provide suitable conditions for ESI. It is demonstrated that sheath-liquid interfacing for protein analysis is adequately robust with protein peak area reproducibilities within 10% RSD. Sheath liquids used for positive ionization of proteins most commonly consist of a mixture of water, an organic solvent and a volatile acid. Optimization of the sheath-liquid composition is an important aspect in order to achieve good protein signals in ESI-MS. Particularly, the type and concentration of organic solvent can significantly affect protein ionization. For instance, for some proteins isopropanol in the sheath liquid can cause substantial enhancement of ionization efficiencies. On the other hand, in some cases the organic solvent might induce denaturation of the protein in the gas phase, as was observed for cytochrome *c* during sheath-liquid CE-MS experiments reported in this thesis.

An intrinsic disadvantage of the use of a sheath liquid in CE-MS is that it dilutes the CE effluent, and thereby reduces sensitivity as ESI-MS behaves as a concentration-sensitive detector. The use of sheathless interfacing for CE-MS, therefore, seems to be an attractive option to obtain higher MS responses and low limits of detection (LODs) in CE-MS. However, the design of a robust sheathless interface that provides adequate electrical contact for both CE and ESI, has proven to be challenging. In this thesis, a new prototype porous tip sprayer for sheathless CE-MS was evaluated for intact protein analysis. In contrast to previous interface designs which often rely on electrical contact outside the capillary, in the new

sheathless interface electrical contact with the BGE is established through the capillary wall which is made porous and conductive. The porous tip interface is an integrated part of the CE capillary, which makes installation and use very straightforward. The absence of sheath liquid indeed led to higher protein MS responses and, particularly, to lower noise levels than obtained with sheath-liquid CE-MS. Using a dedicated nanospray ion source sub-nM LODs for intact proteins were obtained, which was clearly an improvement compared to CE-MS using an optimized sheath liquid. Notably, sheathless interfacing requires a continuous liquid flow emerging from the CE capillary to produce a stable electrospray. In our study, a PEI capillary coating was used to induce a significant and constant EOF.

4. Applicability

The CE-TOF-MS systems described in this thesis provide efficient and reproducible tools for the characterization of biopharmaceuticals. In order to create conditions in which analyte adsorption is minimized, acidic biomolecules (p*I*<7) can be analyzed with the CE-MS system employing the negatively-charged PB-PVS coating using a BGE of medium or high pH. On the other hand, for basic proteins and peptides (p*I*>7) the positively-charged PB-DS-PB and PEI coatings are the most likely choice in combination with a BGE of low pH. The results in this thesis show that the developed CE-MS methods are particularly useful for the characterization of closely-related degradation products, impurities and isoforms. That is, for those situations in which the charge-to-size separation mechanism of CZE can be fully exploited. Especially protein modifications leading to analyte charge differences can be effectively discerned, as they can be efficiently separated from the parent compound. This is illustrated in this thesis for compounds such as llama antibodies, oxytocin, human growth hormone, interferon-β and drug-lysozyme-drug conjugates. When modifications involve sufficiently large changes in size (or mass), like *e.g.* is the case for some of the glycoforms of interferon-β, partial separation might be obtained. In these cases the high mass accuracy and resolution of the TOF-MS still allows unambiguous assignment of the molecular mass of the detected species.

ESI-TOF-MS provides accurate information on the molecular mass of the analyzed proteins and their potential degradation products, impurities and isoforms. It should be noted that no information on the exact nature and intramolecular arrangement of protein modifications is obtained. For quality control in pharmaceutical analysis, however, this normally will be sufficient as information on the character of the product often is available and the number of possible (and likely) modifications is limited. Moreover, as shown for interferon-β, CE-TOF-MS can yield characteristic product profiles which can be used to compare products and/or batches. When profile differences between samples are detected, the TOF-MS data can be used to assign the respective species and pinpoint the structural differences. When unknown components have to be identified, the CE-TOF-MS data on the intact protein should be combined with results obtained with additional protein analysis

methods, like protein digestion followed by peptide or glycan mapping using liquid chromatography-MS/MS.

5. Perspectives

In order to have a better understanding of protein adsorption (and ultimately arrive at even more effective capillary coatings), it would still be interesting to further study the adsorption behavior of proteins on silica and coated surfaces in more detail. Investigating the actual interaction of various proteins (with different size, p*I*, and hydrophobic surface) with the potential coatings under different conditions (high/low pH and ionic strength) would expand our insight of factors affecting protein adsorption. EW-CRDS in principle is a suitable technique for such studies, however, for sensitive detection EW-CRDS is still limited to proteins that absorb light in the visible light range. Potentially, surface plasmon resonance (SPR) could be used, but the synthesis of a silica-like layer on the gold SPR surface seems the major obstacle for the application of this technique. Preliminary experiments at our university indicate that atomic force microscopy (AFM) provides interesting topological data of the surface of coating surfaces and allows monitoring of protein adsorption.

When protein samples of large heterogeneity need to be analyzed, the use of neutrally coated capillaries might be required to enhance resolution. With neutral capillaries, the separation is merely based on the electrophoretic mobility of the compounds and not affected by EOF which could reduce resolution. So, CE-TOF-MS employing capillaries with neutral coatings would be worthwhile to study. However, as these capillaries do not generate an EOF (and thus no liquid flow towards the capillary end), their coupling with sheathless CE-ESI-MS might be challenging. Applying a hydrodynamic pressure at the capillary inlet during CE analysis (to induce a small liquid flow) might solve the problem, but at the expense of a loss in CE resolution.

In this thesis, CE-TOF-MS was successfully applied for the analysis of proteins with molecular weight between 5 and 50 kDa. The study of biopharmaceuticals of much larger size, like *e.g.* monoclonal antibodies (mAbs; 150 kDa), would be very interesting as these take a prominent position in today's protein therapeutics. For these large proteins, the use of mass spectrometers with a high resolution in combination with CE should be considered in order to obtain useful information. Orbitrap or Fourier transform ion cyclotron resonance (FT-ICR) mass analyzers, which provide resolutions above 500,000, would be very suitable for this task. It is sometimes argued that high resolution mass spectrometers could permit protein identification in mixtures without prior separation of the components. It should be noted, however, that with a mass resolution of 500,000 one can only reliably differentiate between a protein and its singly deamidated form when its molecular mass does not exceed 8000. So even with high-resolution mass spectrometers available, separation (*e.g.* by CE) of highly related protein species is essential. When applying FT-ICR-MS as CE detector, its relatively

long duty cycle may compromise the analysis of narrow CE peaks, whereas shorter duty cycles may lead to reduced sensitivity.

As indicated above, the exact nature and position of protein modifications is difficult or impossible to assign based on molecular mass information only. Classical protein digestion followed by peptide mapping can elucidate protein modifications. In case of glycosylation, glycans can be identified by cleavage from the protein followed by LC-MS. These approaches work very well, but require multiple sample handling steps which could affect the integrity of the protein. In this respect, the employment of electron transfer dissociation (ETD)-MS of intact proteins would be very interesting. ETD causes fragmentation of the intact protein mainly resulting in c and z fragment ions, and thus allows elucidation of protein structure, including modifications. It could be interesting to combine high-resolution ETD-MS with the highly efficient protein separations provided by CE in order to obtain detailed structural analysis of proteins and their potential degradation products and isoforms.

With sheathless CE-MS favorable LODs can be obtained. However, the use of sample concentration methods might further improve LODs obtainable with CE-MS. One could consider the use of pH-mediated stacking. Proteins are stacked at the boundary of the sample zone and the BGE, which differ in pH. Such approaches have been used with CE-UV and have led to concentration factors up to 2000 for myoglobin. Protein binding on affinity solid-phase extraction material placed in front of or in the CE capillary could lead to high concentration factors. With this approach, proteins can be selectively concentrated, while the nonbound fraction is removed.

In biopharmaceutical industry, capillary gel electrophoresis (CGE) and capillary isoelectric focusing (CIEF) in combination with UV or fluorescence detection are routinely applied for the characterization of biopharmaceuticals. Combination of these techniques with MS would be highly useful and rewarding. However, unfortunately, the gel polymers and ampholytes used for separation cause significant ionization suppression and ion source contamination. So, strategies to circumvent these problems and improve compatibility of CGE and CIEF with MS should be developed. Recently, the use of a gel removal step was demonstrated in a combination of conventional gel electrophoresis with inductively-coupled plasma (ICP)-MS. Via osmosis, gel polymers were removed from the solution just after separation, while proteins migrated towards the ICP-MS interface. A similar approach could be envisioned for CGE-MS and CIEF-MS. Still, introducing such a step at the end of a CE capillary will be challenging and will most likely affect the separation efficiency, but would significantly enhance the utility of CIEF and CGE in protein analysis.

There are still some interesting application fields for CE-ESI-MS of intact proteins that have hardly been explored. The application of CE-MS for the comparison of profiles of biopharmaceutical biosimilars shows very good potential. The excellent migration time and peak area RSDs provided by the CE-TOF-MS methods described in this thesis, will allow reliable comparisons between different batches and products. Furthermore, the CE-MS

analysis of mAbs, which represent the most significant part of today's biopharmaceuticals, has been hardly explored yet. Many modifications, such as glycosylation, may affect the properties of mAbs. As these modifications may involve a change in net charge and size of the protein, CE-ESI-MS would be a very suitable tool for the characterization of these proteins. Creating gas phase ions by ESI from such high-molecular-weight compounds after a CE separation might require thorough optimization and characterization of separation and ionization parameters, including adaption of MS instrumentation. As ESI-MS of mAbs leads to highly charged species with a broad charge state distribution, LODs for mAbs might be less favorable compared to proteins with a lower molecular weight. So, sensitive detection like obtained with sheathless CE-MS is indicated. It might also be attractive to study noncovalent protein complexes or to perform protein folding studies with CE-ESI-MS. Both the CE separation and ESI can be performed under native conditions, keeping complexes or folding states intact. For protein folding studies, combining CE-ESI-MS results with data from optical detection techniques like wavelength-resolved fluorescence, time-resolved phosphorescence, and/or circular dichroism seems very useful. In this way, complementary information on folding processes would be obtained.

Nederlandse Samenvatting

Summary in Dutch

Biofarmaceutica zijn geneesmiddelen die verkregen zijn uit levende organismen. Zij worden, in tegenstelling tot de meeste conventionele geneesmiddelen, niet geproduceerd door chemische synthese. Aan het eind van de 19de eeuw werden biofarmaceutica geïntroduceerd als een nieuwe manier om ziektes te genezen en voorkomen. De eerste van deze middelen waren vaccins tegen cholera, tyfus en de pest. In de daarop volgende decennia werden peptiden en eiwitten, die waren geïsoleerd en gezuiverd uit dierlijk materiaal, gebruikt als geneesmiddel. Aan het begin van de jaren ’70 van de vorige eeuw groeide de ontwikkeling van biofarmaceutica sterk door de introductie van recombinante DNA technologie. Het eerste eiwitgeneesmiddel dat beschikbaar kwam via biotechnologie was humaan insuline. Sindsdien heeft de productie van eiwitten voor therapeutische doeleinden geleid tot nieuwe behandelingsmethoden voor verscheidene chronische en levensbedreigende ziekten. Tegenwoordig bevat het assortiment van biofarmaceutica onder andere vaccins, therapeutische eiwitten en gentherapeutica.

Zoals voor elk geneesmiddel voor menselijk gebruik moeten biofarmaceutica voldoen aan strenge kwaliteitseisen om de werkzaamheid en veiligheid te garanderen. Vanwege hun complexe structuur en grootte, is de volledige karakterisering van biofarmaceutische eiwitten uitdagend. Bovendien kunnen de ingewikkelde biotechnologische processen resulteren in een productievariabiliteit die van invloed kan zijn op de activiteit. Dit betekent dat meestal een groot aantal analysetechnieken moet worden toegepast om een volledig overzicht te verkrijgen van het gehalte en de samenstelling van een farmaceutisch eiwit. Specifieke en betrouwbare bepaling van onzuiverheden, afbraakproducten of isovormen van biofarmaceutische eiwitten vereisen vaak een scheiding van de componenten voorafgaand aan hun detectie. De karakterisering van biofarmaceutica heeft dus zeker baat bij een aanpak waarin efficiënte scheiding en selectieve detectie “on-line” gecombineerd worden.

Geladen verbindingen, zoals eiwitten, kunnen efficiënt worden gescheiden met behulp van elektroforetische technieken. Polyacrylamide gelelektroforese (PAGE) is een techniek die veel gebruikt wordt voor de analyse van eiwitten. Eén-dimensionale PAGE biedt over het algemeen een efficiënte scheiding die is gebaseerd op eiwitgrootte, maar kan niet worden gebruikt om eiwitten die sterk aan elkaar gerelateerd zijn, zoals afbraakproducten of glycovormen, te onderscheiden. De resolutie kan worden verbeterd met behulp van twee-dimensionale gelelektroforese (2D-GE), maar 2D-GE is arbeidsintensief en heeft een zeer lange analysetijd. Bovendien kan 2D-GE niet on-line worden gekoppeld aan massaspectrometrie (MS) en mist het de mogelijkheid om te kunnen kwantificeren met een hoge nauwkeurigheid.

Capillaire elektroforese is een aantrekkelijke techniek voor efficiënte en snelle scheidingen van eiwitten. Capillaire iso-elektrische focussering (CIEF) wordt momenteel vaak gebruikt om de ladingsheterogeniteit van farmaceutische eiwitten te bepalen. Capillaire gelelektroforese (CGE) wordt toegepast om de aanwezigheid van subeenheden of fragmenten van eiwitten, zoals bij monoklonale antilichamen, te controleren. Door het gebruik van relatief

hoge concentraties van niet-vluchtige amfolyten (CIEF) en gelpolymeren (CGE), is de combinatie van deze technieken met MS echter nogal problematisch. In dit opzicht biedt capillaire zone elektroforese (CZE) betere mogelijkheden. CZE kan eiwitten zeer efficiënt scheiden op basis van hun lading/grootte verhouding. Bovendien kan CZE worden uitgevoerd in waterige buffers, waarbij eiwitten kunnen worden gedetecteerd in hun natieve toestand. Scheidingsparameters kunnen snel worden geoptimaliseerd door het veranderen van de aard, concentratie en pH van het achtergrondelektroliet (BGE), en vluchtige BGE's kunnen worden geselecteerd om de koppeling met MS te vergemakkelijken.

Moleculen met een hoog molecuulgewicht, zoals eiwitten, hebben een relatief lage diffusiecoëfficiënt. Daarom kunnen voor eitwitten in principe zeer smalle pieken met CZE worden verkregen. Echter, een risico bij de analyse van eiwitten met CZE is dat eiwitten aan de capillairwand adsorberen. Door deze adsorptie zal de scheidingsefficiëntie verslechteren en eventueel leiden tot verlies van eiwit door irreversibele adsorptie. Eiwitadsorptie kan worden voorkomen door gebruik te maken van BGE's met een zeer hoge of lage pH of met een hoge ionsterkte. Deze benaderingen zijn echter beperkt in toepasbaarheid als gevolg van problemen met eiwitstabiliteit en hun incompatibiliteit met MS-detectie. Over het algemeen wordt eiwitadsorptie in CZE voorkomen door de capillairwand te "coaten". Een elegante en eenvoudige manier om effectieve coatings te maken is door het aanbrengen van geladen polymeren op het silica capillairoppervlak. Deze coatings kunnen simpel worden aangebracht door het capillair te spoelen met een oplossing van een positief geladen polymeer die elektrostatisch wordt geadsorbeerd aan de negatief geladen capillairwand. Meer stabiele CZE systemen kunnen worden verkregen door het aanbrengen van meerdere lagen (twee of meer) van polymeren met tegengestelde lading. Door de lading van de laatste polymeerlaag te combineren met een BGE van de juiste pH kunnen zeer efficiënte CZE systemen worden verkregen die geschikt zijn voor de analyse van zure of basische eiwitten.

In de afgelopen decennia heeft MS zich ontwikkeld tot een van de meest populaire detectietechnieken voor de karakterisering van eiwitten. Met electrospray-ionisatie (ESI) kunnen eiwitten vanuit een oplossing als ionen in de gasfase worden gebracht. ESI van volledige eiwitten resulteert in ionen met meerdere ladingen die doorgaans *m/z*-waarden tussen de 500 en 3000 hebben. Dit ligt binnen het meetbereik van de meeste commercieel beschikbare massa-analysatoren. Molecuulmassa's van eiwitten worden verkregen door deconvolutie van de ESI massaspectra. ESI is een zachte ionisatiemethode waarbij geen eiwitfragmentatie optreedt en die zelfs de conformatie van de geanalyseerde eiwitten ongewijzigd kan laten. Dit kan belangrijk zijn wanneer de structuureigenschappen van intacte eiwitten, zoals bijvoorbeeld biofarmaceutica, onderzocht worden.

De koppeling van CZE met MS is een potentieel krachtige benadering voor de analyse van eiwitten. CZE-MS kan niet alleen zeer nuttig zijn voor de bepaling van zuiverheid en stabiliteit, maar ook voor de karakterisering van sterk op elkaar gelijkende eiwitten. Momenteel worden CZE en MS het meest gekoppeld via ESI met behulp van een

zogenaamde ‘sheath-liquid’ interface. CZE en ESI vereisen gesloten stroomkringen met een gemeenschappelijke elektrode aan het uiteinde van het capillair. De sheath liquid maakt de nodige elektrische contacten mogelijk. Voor de analyse van intacte eiwitten met CZE-ESI-MS is het nodig om massa-analysatoren met een hoge resolutie en nauwkeurigheid, zoals een vluchttijd (TOF) analysator, te gebruiken. De sterke technologische ontwikkelingen in TOF-instrumentatie en de brede commerciële beschikbaarheid van TOF-MS instrumenten tijdens het laatste decennium bieden goede mogelijkheden voor de CE-MS analyse van intacte eiwitten.

Bij de start van dit project was de toepasbaarheid van CE-TOF-MS voor de analyse van biofarmaceutica nog nauwelijks onderzocht. Voor het vaststellen van de potentie van dit systeem op dit gebied zijn efficiënte en reproduceerbare eiwitscheidingen nodig. Vandaar dat de toepasbaarheid van capillairen met niet-covalente coatings op basis van geladen polymeren bestudeerd is. Eerder werk heeft uitgewezen dat coatings van Polybreen-(polyvinyl sulfonaat) (PB-PVS) zeer geschikt zijn voor de analyse van zure eiwitten. Echter, coatings met meer lagen voor de CE-MS analyse van basische eiwitten waren nog niet systematisch geëvalueerd. In dit proefschrift ligt daarom de nadruk op de ontwikkeling van CE methoden voor basische eiwitten, waarbij in het bijzonder aandacht is besteed aan positief geladen Polybreen-dextraansulfaat-Polybreen (PB-DS-PB) coatings. De prestaties en stabiliteit van de coating voor de analyse van basische eiwitten zijn geëvalueerd bij neutrale en lage pH. De geschiktheid van de coating voor CE-TOF-MS is bestudeerd, waarbij veel aandacht is besteed aan de compatibiliteit tussen CE en MS. De koppeling van CE met MS is tot stand gebracht via een sheath-liquid interface, waarbij belangrijke condities voor de interfacing zijn onderzocht. Een nadeel van dit type interface is dat de sheath liquid de vloeistofstroom die uit het capillair komt verdunt wat leidt tot verminderde gevoeligheid. Om dit te vermijden is een “sheathless” interface voor de analyse van intacte eiwitte met CE-MS geëvalueerd en vergeleken met sheath-liquid CE-MS. De toepasbaarheid van de ontwikkelde CE-TOF-MS systemen is bestudeerd door het analyseren van diverse farmaceutische eiwitten, waarbij aandacht is besteed aan aspecten zoals zuiverheid, stabiliteit en heterogeniteit.

In **hoofdstuk 1** van dit proefschrift zijn de uitgangspunten en het doel van het uitgevoerde onderzoek beschreven. Een overzicht van het gebruik van CE-MS op het gebied van intacte eiwitanalyse is gepresenteerd in **hoofdstuk 2**. Sectie 2.1 behandelt de CE scheidingstechnieken, coating- en preconcentratietechnieken die zijn toegepast. Bovendien worden verschillende CE-MS interfaces en MS-analysatoren beschreven die gebruikt worden voor eiwitanalyse. De toepasbaarheid van CE-MS in eiwitanalyse wordt geïllustreerd met voorbeelden uit gebieden zoals “biomarker discovery”, “glycoprotein profiling” en proteomics. In sectie 2.2 worden de technologische ontwikkelingen en nieuwe toepassingen van CE-MS voor de analyse van intacte eiwitten in de periode 2007-2010 besproken.

In **hoofdstuk 3** is de effectiviteit van positief geladen polymeer-coatings voor het minimaliseren van de adsorptie van basische eiwitten bestudeerd met behulp van "evanescent-wave cavity ring-down spectroscopie" (EW-CRDS). Adsorptie van cytochroom *c* op silica gecoat met een laag PB of met een drievoudige laag van PB-DS-PB, is bestudeerd en vergeleken met silica zonder coating. Uit directe analyse van de silica-oppervlakken door EW-CRDS is gebleken dat beide coatings eiwitadsorptie effectief verminderen. Significante adsorptie is alleen waargenomen voor eiwitconcentraties boven 400 µM, waarbij de PB-DS-PB coating het meest effectief en stabiel bleek te zijn. CE analyses van cytochroom *c* zijn ook uitgevoerd met en zonder coating. Bestudering van de "electroosmotic flow" (EOF) en piekoppervlakken van de eiwitten toont een sterke reductie van irreversibele eiwitadsorptie door de positief geladen coatings. Aan de hand van de elektroforetische mobiliteit en piekbreedte van cytochroom *c* is evenwel reversibele eiwitadsorptie aan de PB coating aangetoond.

Hoofdstuk 4 beschrijft het gebruik van de niet-covalente PB-DS-PB coating voor de analyse van intacte basische eiwitten met CE-UV. Karakteriseringstudies van de coating tonen aan dat een stabiele coating kan worden verkregen die een pH-onafhankelijke en zeer reproduceerbare EOF genereert. De PB-DS-PB coating is geëvalueerd met BGEs van Tris-fosfaat van verschillende pH met behulp van vier basische modeleiwitten. De variabiliteit van de migratietijd voor de eiwitten was minder dan 0,85%, en schotelgetallen hoger dan 125.000 zijn verkregen, waardoor enkele onzuiverheden in de modeleiwitten konden worden gescheiden van de hoofdcomponenten. De toepasbaarheid van het CE-UV systeem is onderzocht met de profilering van immunoglobuline G_1 bij neutrale pH en de analyse van lama-antilichamen en hun bindingsproducten bij lage pH. Ten slotte zijn de mogelijkheden van het gebruik van de PB-DS-PB gecoate capillairen voor CE in combinatie met TOF-MS verkend aan de hand van de karakterisering van een lama-antilichaammonster.

De CE-MS analyse van basische intacte eiwitten met behulp van capillairen gecoat met PB-DS-PB is bestudeerd en verder geoptimaliseerd in **hoofdstuk 5**. De coating bleek volledig compatibel met MS-detectie; er werden geen achtergrondsignalen en onderdrukking van ionisatie waargenomen. De samenstelling van de sheath-liquid en BGE is geoptimaliseerd met behulp van modeleiwitten. Dissociatie van de heemgroep in cytochroom *c* werd waargenomen bij hoge concentraties isopropanol in de sheath-liquid. Onder geoptimaliseerde omstandigheden konden onzuiverheden, die aanwezig zijn in het mengsel, worden gedetecteerd en geïdentificeerd met behulp van de verkregen accurate massa's. De selectiviteit van het CE-MS systeem is onderzocht door de analyse van geacetyleerd lysozym. Acht sterk verwante verbindingen, geïdentificeerd als niet-geacetyleerd lysozym en lysozym met verschillende acetyleringsgraden, konden worden onderscheiden. Het CE-MS systeem had zowel een goede reproduceerbaarheid als goede lineariteit. Detectielimieten voor eiwitten met het CE-MS systeem lagen tussen de 11 en 19 nM.

De toepasbaarheid van CE-TOF-MS met niet-covalente capillaire coatings voor de analyse van biofarmaceutische producten wordt gedemonstreerd in **hoofdstuk 6**. PB-PVS en PB-DS-PB coatings zijn gebruikt om de adsorptie van eiwitten en peptiden tot een minimum te beperken en goede scheidingen te behalen. De mogelijkheden van de CE-MS systemen om afbraakproducten te karakteriseren is onderzocht door de analyse van de geneesmiddelen recombinant humaan groeihormoon (rhGH) en oxytocine, die waren blootgesteld aan langdurige opslag, warmte en/of verschillende pH-waarden. Modificaties van rhGH en oxytocine, zoals deamidaties en oxidaties, konden worden toegewezen op basis van de verkregen CE scheiding en accurate massa. Recombinant humaan interferon-β-1a (rhIFN-β) is gebruikt om de geschiktheid te bepalen van de CE-MS methode voor de evaluatie van glycaanheterogeniteit van farmaceutische eiwitten. Analyse van dit geglycosyleerde eiwit resulteerde in een cluster van pieken die afkomstig zijn van tenminste tien glycovormen die bleken te verschillen in siaalzuur- en hexose-N-acetylhexosamine-samenstelling. De conclusie is dat CE-TOF-MS het mogelijk maakt om verwante eiwitten te onderscheiden.

Hoofdstuk 7 beschrijft een studie naar de prestaties van een interface voor sheathless CE-MS van intacte eiwitten. Capillairen met een poreus uiteinde zijn in een roestvrijstalen naald geplaatst die is gevuld met een statische vloeistof. Deze is in eerste instantie in een conventionele ESI bron geïnstalleerd. Zeer reproduceerbare en efficiënte scheidingen van vier modeleiwitten zijn verkregen met behulp van een positief geladen polyethyleenimine (PEI) coating. Sheath-liquid CE-MS met dezelfde capillairen en een sheath-liquid van isopropanol-water-azijnzuur resulteerde in iets lagere of gelijke analietsignalen. De ruisniveaus waren echter hoger met sheath-liquid CE-MS, waardoor de detectielimieten een factor 6-20 hoger waren dan vergeleken met sheathless CE-MS. De signalen van de eiwitten in sheathless CE-MS kon worden verbeterd met behulp van een “nanoESI” bron en toevoeging van 5% isopropanol aan de BGE. Dit leidde tot detectiegrenzen in het sub-nM gebied, wat 50 tot 140 keer meer gevoelig is dan met sheath-liquid CE-MS gebruikmakend van dezelfde capillairen.

In **hoofdstuk 8** is sheathless CE-TOF-MS gebruikt om geneesmiddel-lysozym conjugaten te karakteriseren. Om adsorptie van lysozymconjugaten aan de capillaire wand te voorkomen, is een PEI coating gebruikt in combinatie met een BGE van lage pH. Geneesmiddel-lysozym producten zijn gemaakt door eerst BOC-L methionine hydroxysuccinimide ester (BOCmet) te koppelen aan lysineresiduen van lysozym, gevolgd door conjugatie met de kinase-remmers LY364947, erlotinib en Y27632 via een platinalinker. CE-TOF-MS geeft een goede scheiding en identicatie van de verschillende reactieproducten. Uit de TOF-MS gegevens bleek dat alle preparaten, naast niet-gemodificeerd lysozym en lysozym met een BOCmet modificatie, ook lyzozym bevatten met één of twee gebonden geneesmiddelmoleculen. Op basis van de relatieve piekoppervlakken is een kwantitatief conjugaatprofiel verkregen voor elk product. Uit deze resultaten is een totale belading van 0,52, 0,59, en 0,42 mol geneesmiddel per mol eiwit berekend voor respectievelijk de LY364947-, erlotinib- en Y27632-lysozym preparaten.

Hoofdstuk 9 bevat algemene conclusies en opmerkingen over de CE-MS systemen die ontwikkeld zijn voor de analyse van biofarmaceutica. Ook toekomstperspectieven en aanbevelingen voor verder onderzoek worden gepresenteerd.

Curriculum vitae

Rob Haselberg werd geboren op 2 december 1982 te Lochem. Na het behalen van zijn HAVO diploma aan het Staring College te Lochem begon hij in 2000 met de studie Algemene Chemie aan Saxion Hogeschool IJselland te Deventer. Deze studie werd afgerond met een onderzoeksstage bij de "Chemical Analysis Group" van de Universiteit Twente in Enschede onder begeleiding van dr. Christel Hempen, dr. Martin Vogel en prof. dr. Uwe Karst. De titel van het stageverslag was 'post-column derivatization of cytochrome *c* digests' en de hierin beschreven resultaten waren tevens de basis voor Rob's eerste onderzoeksartikel. Na het behalen van zijn bacherlordiploma met lof in juli 2004, werd begonnen met de masteropleiding Analytical Sciences aan de Vrije Universiteit Amsterdam. Als afsluiting van deze opleiding werd wederom een onderzoeksstage bij de Chemical Analysis Group van de Universiteit Twente gedaan. Onder begeleiding van dr. Martin Vogel en prof. dr. Uwe Karst werd gewerkt aan het project 'Surface modification of fluorescent nanoparticles for applications in bioanalytical assays'. Het masterdiploma werd in april 2006 tevens met lof behaald. Aansluitend was hij, van april 2006 tot en met juni 2010, als assistent in opleiding verbonden aan de vakgroep Biomedische Analyse van de Universiteit Utrecht onder begeleiding van prof. dr. G.J. de Jong en dr. G.W. Somsen. Het onderzoek, dat werd gefinancierd door een VIDI-beurs van STW gehonoreerd aan dr. G.W. Somsen, had als doel het ontwikkelen van methoden gebaseerd op capillaire electroforese-massaspectrometrie voor de analyse van biofarmaceutica. Onderzoeksresultaten werden gepresenteerd op zowel nationale als internationale congressen in de vorm van posterpresentaties en lezingen. Sinds september 2010 werkt hij als onderzoeker bij de vakgroep Biomedische Analyse. Zijn huidige onderzoek richt zich op de karakterisering van een nieuw CE-MS interface en de toepasbaarheid daarvan voor de analyse van monoclonale antilichamen.

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