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Review

CE-MS for the analysis of intact proteins 2010–2012

Since its introduction in 1987, CE-MS has become an increasingly important technique for the analysis of biomolecules. Since our previous update on CE-MS methods within the field of intact protein analysis (*Electrophoresis* 2011, *32*, 66–82), a variety of interesting methodological improvements and applications have been reported in literature. Therefore, this article presents an overview of the development and application of CE-MS for intact protein analysis as published between June 2010 and June 2012. The article is divided in sections that treat CE coupled to MS through ESI, MALDI, and ICP ionization, respectively. In the section about CE-ESI-MS, technological developments with respect to CE-MS interfacing, prevention of protein adsorption, and chip-based CE-MS are treated in more detail. Novel interfacing strategies and the development of improved capillary coating strategies appeared to be the major developments. Furthermore, in all sections, the applicability of CE-MS for intact protein analysis is demonstrated by representative examples, including important developments in the fields of biopharmaceutical characterization and the analysis of proteins in biological samples. Finally, some general conclusions and future perspectives are given.

Keywords:
Capillary electrophoresis / Electrospray ionization / Inductively coupled plasma ionization / Intact proteins / Matrix-assisted laser-desorption ionization
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1 Introduction

CE-MS combines high separation efficiencies 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 high resolving power. For example, CE-MS has been applied in fields such as proteomics [1], metabolomics [2], and forensic science [3], as well as for pharmaceutical [4] and food analysis [5].

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. CE-MS exhibits interesting possibilities for the characterization of intact proteins, providing information on protein quality including isoforms, degradation products, and impurities [6, 7]. 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. On the other hand, MS detection can provide highly useful information on the molecular weight and folding state of analyzed protein species [8–10]. Therefore, the combination of CE and MS is a strong tool for the characterization of intact proteins.

Coupling of CE and MS is most commonly carried out by ESI using either sheath liquid, sheathless, or liquid junction interfaces [11, 12]. Although CE-ESI-MS coupling is less straightforward than, for example, LC-ESI-MS, it is now routinely applied in a significant number of laboratories. In ESI, proteins become multiply charged upon desolvation resulting in a charge state distribution, which conveniently allows their detection in m/z ranges of 500 to 3000. CE can also be combined with MS by using MALDI [12] or ICP ionization [13]. These two coupling strategies are less frequently applied, but still have some specific differences with respect to ESI. With MALDI singly or doubly charged protein molecules are formed allowing direct determination of molecular mass. Moreover, MALDI is more tolerant to sample constituents like buffers and salts than ESI, and, therefore, nonvolatile BGEs can be used for CE-MALDI-MS. On the other hand, MALDI cannot be performed on-line and its performance depends on the nature of the matrix and the quality of the crystallization of the matrix and analytes. With ICP ionization,

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Abbreviations: **AGP**, α-1-acid glycoprotein; **AuNP**, gold nanoparticle; **DS**, dextran sulfate; **EIE**, Extracted ion electropherogram; **EPO**, erythropoietin; **PB**, Polybrene; **SATA**, *N*-succinimidyl-*S*-acetylthioacetate

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the sample and buffer are passed through a plasma torch (>5000 K) and all compounds are broken down to in their charged elements, typically in the M^+ state. Elements like, carbon, oxygen, and nitrogen cannot be used for detection, since they are also abundantly present in the BGE. However, with CE-ICP-MS highly selective detection of, for example, sulfur-, phosphorus-, and metal-containing proteins can be performed.

A comprehensive overview of publications reported between 1987 and 2007 on CE-MS for the analysis of intact proteins was published in May 2007 [6]. An update of this review, covering the period from 2007 till June 2010, was published in January 2011 [7]. The present paper is an update of the former reviews covering the literature in the period July 2010 to June 2012. The paper is confined to CE-MS studies in which intact protein molecules are separated, i.e. in which proteins are not deliberately broken down into peptides or derivatized prior to analysis. The review starts first with a section on the combination of CE with ESI-MS, focusing on technological advances and selected applications. Subsequently, the combination of CE with MALDI-MS and ICP-MS is shortly discussed. Finally, some general conclusions and future perspectives are given.

2 CE-ESI-MS

2.1 Technological developments

2.1.1 CE-ESI-MS interfacing

CE-MS is most commonly performed using a sheath liquid ESI interface, since this interface is well characterized and commercially available. The constituents of the BGE are an important aspect in CE-ESI-MS. Volatile buffers of low concentration are preferred for sensitive MS, but might not provide optimum CE separations. It has been frequently demonstrated that efficient protein separation can be obtained with BGEs of formic or acetic acid. Notably, ammonia, often added to modify the pH, might result in ionization suppression thereby lowering the achievable sensitivity. Due to the dilution of the capillary effluent by the sheath liquid, the detection sensitivity can be even further compromised [14]. Moreover, multiple charging of proteins occurring during ESI distributes the overall signal intensity over many charge states, thereby even further increasing the achievable detection limits for intact proteins. Therefore, in CE-MS of intact proteins there is a definite requirement for increased sensitivity. To improve the sensitivity, interfaces with no or limited sheath flow could be employed. As effluent volume flow rates are low, the initial droplets formed during the electrospray process are small, leading to more efficient ionization (i.e. nanospray) [15]. This approach also allows the ESI spray tip to be positioned closer to the MS inlet and, thereby, ion sampling efficiencies are improved [15, 16].

In our previous review, we indicated two novel interfaces, i.e. the porous tip design of Moini [17] and the junction-at-the-tip design of Chen et al. [18], for the analysis of intact proteins. Over the past two years, the applicability of these interfaces for intact protein analysis was further investigated. The sheathless CE-MS interface developed by Beckman Coulter, which is based on the design of Moini, gained quite some attention for several applications (e.g. [14, 19–22]). It makes use of an etched (conductive) terminating end of the fused-silica separation capillary to close the electrical circuit of the CE. This interface was evaluated for the analysis of intact model proteins by Haselberg et al. and its performance was compared with conventional sheath liquid interfacing [14]. With the latter approach LODs between 30 and 100 nM were obtained, whereas sheathless CE-MS provided LODs in the low-nM range. Subsequently, a significant improvement for sheathless CE-MS could be made by the use of a nanospray interface and the addition of organic solvent to the BGE. LODs between 0.5 and 1.3 nM were established, which was 50- to 140-fold better than sheath-liquid interfacing using the same capillary. The gain in sensitivity was the result of both reduced noise levels and increased analyte responses as obtained with sheathless CE-MS compared to sheath liquid CE-MS. In two additional papers, the authors demonstrated the applicability of the sheathless CE-MS system, by analyzing protein-drug conjugates and modified nanobodies [23, 24] (see Section 2.2.1). The same interface was also used by Moini to study metal stochiometry and metal displacement of intact proteins [25] (see Section 2.2.3).

Over the past years, the group of Chen has put significant effort in exploring the utility of the junction-at-the-tip CE-MS interface [18, 26–30]. With this interface the interior of a hollow needle tip forms a flow-through microvial at the end of the separation capillary. A chemical modifier solution introduced at a low flow rate carries the analyte from the capillary terminus to the site of ESI even when there is no bulk flow in the separation capillary, while minimizing dilution of the analyte. Zhong et al. used this interface for CIEF-ESI-MS of model proteins, thereby proposing injection, focusing, and mobilization strategies for both uncoated and neutrally coated capillaries [30]. When using uncoated capillaries glycerol was added to the ampholytes-containing BGE to achieve the required reduction of the EOF for efficient CIEF. To enable focusing a part of the capillary was used as catholyte reservoir (Fig. 1Ai). When neutrally coated capillaries were used the flow-through microvial of the CE-MS interface itself served as catholyte reservoir (Fig. 1Aii) since the coating was unstable at high pH. With both capillary types proteins were electrophoretically mobilized by applying an acidic modifier solution (methanol/water/acid; 50:48:2, v/v/v) after focusing. With electrophoretic mobilization amphoteric species are selectively mobilized and this assures that almost no glycerol is introduced into the ion source, where it can cause significant ionization suppression. Good separation efficiencies of model proteins with both uncoated (Fig. 1Bi) and coated capillaries (Fig. 1Bii) were achieved after careful optimization.

Unfortunately, in the approach of Zhong et al. ampholytes are still introduced simultaneously with the sample into the mass spectrometer. Since these compounds are not

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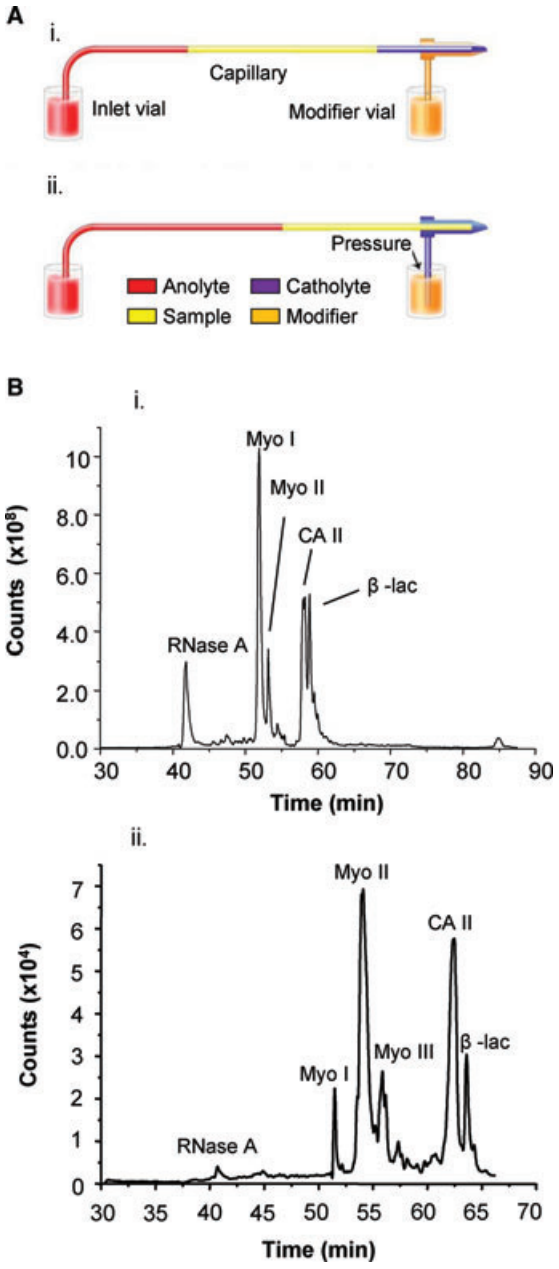


Figure 1. (A) Developed approaches for on-line CIEF-ESI-MS. (i) "Sandwich" injection method for bare-fused silica capillaries. The modifier vial starts to be pressurized to deliver acidic modifier solution when the focused sample zone gets close to the capillary outlet. (ii) Method applied with neutrally coated capillaries. Before separation starts, the modifier delivery capillary is prefilled by catholyte solution with one end connected to the modifier vial. The pressurized modifier vial delivers basic catholyte solution to the microvial during the focusing stage and acidic modifier solution to the microvial during the mobilization stage. (B) Total ion electropherogram for a protein mixture separated by CIEF with electrophoretic mobilization in a (i) bare-fused silica and (ii) a neutrally coated capillary. Legend: RNase A, ribonuclease A; Myo, myoglobin; CAII, carbonic anhydrase II; β-lac, β-lactoglobulin. Reprinted with permission from [30]. Copyright 2011 American Chemical Society.

volatile they may result in background signals and ionization suppression. Wang et al. developed a system that allows detailed protein identification and prevents ampholytes from entering the ion source [31]. CIEF for protein separation is combined with an immobilized enzyme reactor for protein digestion and RP LC for subsequent peptide analysis. In the first stage proteins are separated by CIEF after which they are trapped in a sample loop. Since this was done in an on-line fashion, a porous segment was included between the separation part of the capillary and the sample loop in order to close the electrical circuit. The trapped protein fraction (containing ampholytes) was subsequently transferred to a second porous segment that acted as a dialysis membrane. Here, the pH of the solution was adjusted for efficient trypsin digestion in the immobilized enzyme reactor. After digestion, the peptides were captured on a trap column, whereas the ampholytes were flushed to waste. Next, the peptides were separated using LC and detected with MS. While the digest from the first fraction was analyzed with LC-MS, the second fraction was collected and digested. To evaluate the performance of the system, a 4-protein mixture was analyzed, and sequence coverages between 8 and 54% were obtained. Furthermore, the system was successfully applied for the analysis of proteins extracted from *Escherichia coli*, allowing positive identification of 101 proteins.

2.1.2 Preventing protein adsorption

When using bare-fused silica capillaries for intact protein analysis, CE separation efficiencies, and migration time repeatability may be compromised due to protein-wall interactions. In order to avoid protein adsorption to the inner surface of the capillary and to improve CE performance, coating of the silica surface has shown to be an effective option [32, 33]. In time, various capillary coatings for CE-MS have been proposed and their usefulness for intact protein analysis has been shown.

Most CE-MS studies of intact proteins reported in 2010–2012 apply coatings to obtain highly efficient protein separations. Positively charged coatings based on Polybrene (PB) [25], multilayers of PB, dextran sulfate (DS), and PB [34, 35], polyethyleneimine [14, 23, 24], and *N*,*N*-dimethylacrylamide-ethylpyrrolidine methacrylate [36, 37], are most commonly applied in combination with low-pH BGEs for the analysis of both acidic (pI < 7) and basic proteins (pI > 7). Under these separation conditions, both the proteins and the capillary wall are positively charged, avoiding protein–wall interactions and providing highly efficient separations. These charged coatings generate an appreciable and constant EOF toward the capillary outlet when a reversed polarity is applied, also enabling adequate and reproducible CE-MS interfacing. Moreover, the use of a low-pH BGE is favorable for ESI-MS in positive ionization mode. The disadvantage of a high EOF is that the analyte resolution can be limited, since this is inversely proportional to the sum of the electroosmotic and analyte mobility [38]. Therefore, in some instances neutral

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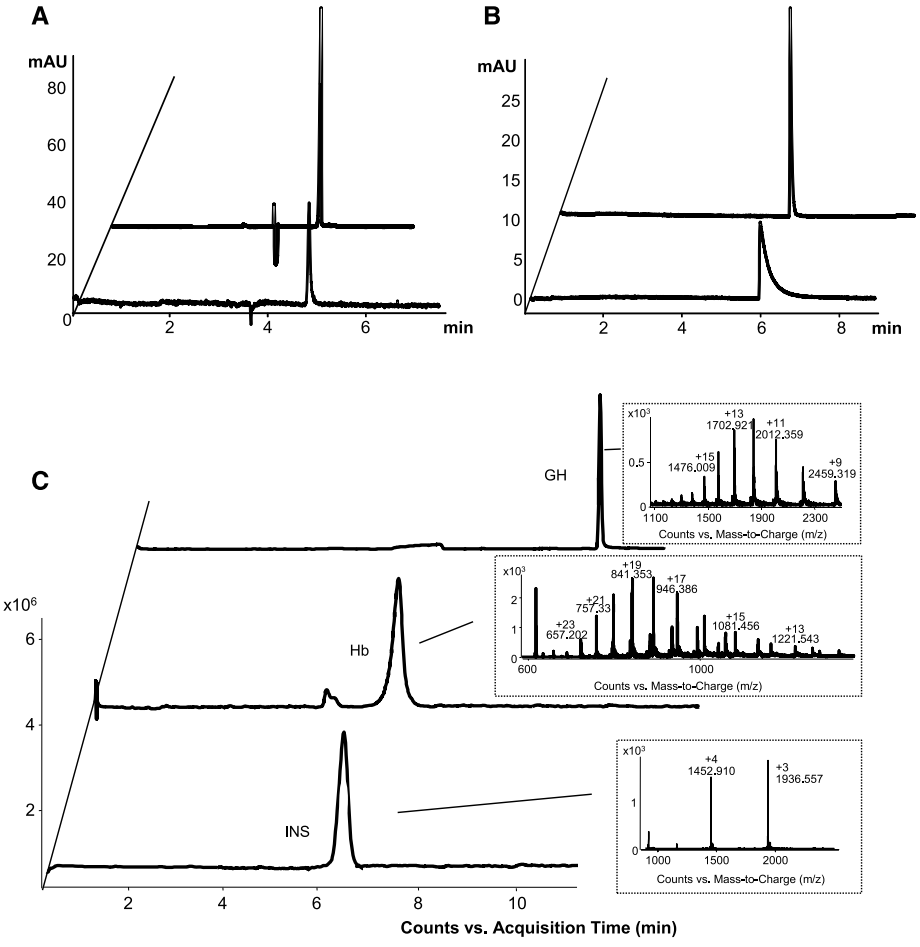


Figure 2. CE-UV electropherogram of insulin obtained with (A) alkaline BGE and (B) acidic BGE. Upper trace: BGE containing 10% ACN. Lower trace: BGE without the addition of ACN. (C) CE-MS electropherograms and extracted mass spectra obtained for insulin (INS) analyzed with alkaline BGE containing 10% ACN, hemoglobin (Hb) analyzed with alkaline BGE without ACN, and growth hormone (GH) analyzed with acidic BGE containing 20% ACN [40]. Copyright Wiley-VCH Verlag GmbH & Co. Reproduced with permission.

capillary coatings have been applied (Table 1). For example, Taichrib et al. recently compared the use of several neutral coatings (dynamic polyacryl amide, covalent polyacryl amide, and thermally immobilized galactomannans guaran and locust bean gum "copolymer") and positively charged coatings (polyethyleneimine, cationic polyacrylamide, and *N,N*-dimethylacrylamide-ethylpyrrolidine metacrylate) for the analysis of model proteins as well as erythropoietin (EPO) with sheath liquid CE-MS using low-pH BGEs [39]. The neutral coatings all resulted in higher separation resolution when compared to the positively charged coatings, on the expense of longer analysis times (factor 2 in some cases). Moreover, it was demonstrated that the nebulizer gas, commonly used in sheath liquid interfacing, significantly increased the EOF of the neutral-coated capillaries due to suction. This shortened the protein's migration time and, when the suction effect is too strong, could have a negative effect on the separation efficiency. From the results presented by the authors it is concluded that the electroosmotic mobility should be slightly higher and opposite to the electrohoretic mobility of the analyte for the highest separation efficiency. However, this approach may lead to very long analysis times and tuning of the EOF with respect to protein mobility is often not feasible. Therefore, neutral coatings with virtually zero EOF provide a good alternative. With this approach, the separation is merely dominated by the analyte mobility providing a good separation resolution within a reasonable time frame.

Often it is assumed that only electrostatic interactions cause protein adsorption, but also hydrophobic interactions and/or protein conformational changes can be involved in this process. Therefore, it can be beneficial to add organic solvents to the BGE in order to prevent protein–wall interactions. Staub et al. used CE-UV to investigate the influence of the addition of ACN to BGEs of low and high pH for the analysis of insulin, human growth hormone, and hemoglobin [40]. They observed that at alkaline pH, when both the capillary wall and proteins are negatively charged the addition of ACN did not lead to an increase of protein peak area. Apparently, no detectable protein adsorption did occur under these conditions. Still, the addition of organic solvent did slightly improve the separation efficiency (Fig. 2A). At low pH the effect of the organic solvent was more pronounced (Fig. 2B), significantly increasing the observed peak area and the separation efficiency. It was concluded that a generic approach is difficult to develop, so the effect of the organic solvent should be investigated on a case-by-case basis. However, it appears that rigid proteins (insulin and human growth hormone) in general benefit from the addition of organic solvent

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to the BGE, whereas for flexible proteins (hemoglobin) it negatively affects the results. Using optimized conditions CE-MS experiments for each individual protein were subsequently performed. The highest peak areas and plate numbers for insulin, human growth hormone, and hemoglobin were obtained with an alkaline BGE containing 10% ACN, an acidic BGE containing 20% ACN, and an alkaline BGE without organic solvent, respectively (Fig. 2C).

2.1.3 Chip-based electrophoresis

Over the last years, there has been a growing interest in the development of chip-based electrophoretic systems for protein analysis [41], as they may increase analysis speed and performance. Sikanen et al. used microchips made through UV-embossing and adhesive bonding [42]. A sheath-flow ESI interface was monolithically integrated with the UV-embossed separation channels. Separations were performed in noncoated channels using a BGE of ammonium acetate (pH 7.0) containing methanol. Although both the channel wall and most of the test proteins were negatively charged still relatively broad and tailing peaks were obtained suggesting significant protein–wall interactions. This is also clear from the migration time repeatability, which decreased with the number of consecutive injections (3.3% RSD for three consecutive runs and 10.7% RSD for eight consecutive runs). However, fast (<5 min) and baseline separations are obtained for the model proteins used. Thus, the possibility of rapid analysis of intact proteins with such a simple setup can become a valuable screening technology before more detailed analysis is performed.

2.2 Applications

An overview of recent CE-ESI-MS applications is provided in Table 1. Some typical examples are outlined in the following paragraphs.

2.2.1 Biopharmaceuticals

Over the past years, biopharmaceuticals have gained increasing attention as a way to improve therapy for certain diseases. Many biopharmaceuticals are glycoproteins, carrying oligosaccharides that are attached posttranslationally. Consequently, glycosylation is a major source of protein heterogeneity. Recently, quite some efforts have been made to characterize glycosylated biopharmaceuticals using CZE-ESI-MS. Recombinant human interferon-β-1a [35] and recombinant human EPO [39, 43, 44] were analyzed by CE-MS in order to distinguish different protein glycoforms. It was demonstrated that most efficient separations of intact glycoforms are obtained when using low-pH BGEs (≤pH 3) in combination with, as indicated in Section 2.1.2, coatings that minimize or cancel out EOF, thereby maximizing effective mobility differences between protein isoforms. An efficient CE separation of EPO combined with high-resolution MS has been published by Taichrib et al. EPO was analyzed using a neutral capillary coating and a BGE of 1 M acetic acid [43]. The separated species were detected with a high-resolution ($Rs > 40\,000$) TOF mass spectrometer. Due to the strongly reduced EOF, a highly efficient protein isoform separation was obtained where even species that differed one neutral sugar unit could be (partially) separated (Fig. 3A). The use of the high-resolution TOF mass spectrometer, compared to a standard TOF-MS, enabled the detection of isotope distributions of all isoforms (Fig. 3B). The isotope distributions enabled the detection of overlapping isoforms differing merely deamidations or cleavage of disulfide bridges.

The group of Neusüß has also put effort in developing chemometrical approaches for the data analysis of glycoform profiles [36, 44], which is an interesting and increasingly important topic. More and more biopharmaceuticals come off patent and as a result, a significant amount of so-called biosimilars and biogenerics become available. In order to compare these products to the original one, especially when they are complex glycoproteins, data analysis is definitely required. Neusüß and coworkers developed a statistical approach to differentiate between various EPO preparations on basis of CE-MS data [44]. Relative peak areas of selected intact EPO isoforms were used as variables in principal component analysis and hierarchical agglomerative clustering. Both approaches appeared to be suitable for the clear differentiation of EPO preparations, differing in manufacturer, production cell line, and/or batch number. Even closely related preparations were distinguished on the basis of the combined information on the antennarity, the sialoform, and the acetylation of the observed isoforms.

Next to glycoproteins, a variety of other clinically relevant proteins have been analyzed with CE-ESI-MS. Staub et al. used CZE-MS to characterize insulin [45]. The protein was analyzed on a bare-fused silica capillary in combination with a high-pH BGE containing 10% ACN. A multiple injection strategy was developed as a way to incorporate an internal standard in their analysis. First a plug of insulin standard was injected (~0.7% of the capillary volume) followed by a large plug of BGE (~9% of the capillary volume). Subsequently, an insulin sample of interest was injected and the separation started. Two peaks, both corresponding to insulin, were obtained of which the first could be used to quantify the second. The methodology was validated according to international guidelines and was successfully applied to the analysis of insulin formulations obtained from regular and parallel markets.

Haselberg et al. used CZE-ESI-MS to characterize three different biopharmaceuticals containing degradation products or naturally occurring isoforms [35]. For this purpose, they used capillaries coated with a double layer of PB and poly(vinylsulfonic acid) or a triple layer of PB-DS-PB. Recombinant human growth hormone was analyzed at high pH

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Table 1. CE-ESI-MS for intact protein analysis

Analytes[a)]	Matrix	BGE/ampholytes	Capillary coating[b)]	Mass analyzer[c)]	Remarks	Ref.
Capillary zone electrophoresis						
Autoinducer inactivation enzyme A	Aqueous solution	10 mM ammonium acetate containing 0.1% PB (pH 6.9)	PB	QTOF	Investigation of metal stochiometry and displacement	[25]
CAII, ins, lys, RNase A	Aqueous solution	100 mM acetic acid (pH 3.1)	PEI	TOF	Comparison sheathless and sheath liquid CE-MS	[14]
Cyt c, myo, ubi, β-LGA, β-LGB	Aqueous solution	20 mM ammonium acetate with 40% methanol (pH 7.0)	No coating	IT	Chip-based CE-MS/new chip material	[42]
Cyt c, lys, RNase A, α-CT	Aqueous solution	50 mM acetic acid (pH 3.0)	PB-DS-PB	TOF	System optimization and performance	[34]
EPO	Pharmaceutical formulations	1 M acetic acid	Linear polyacrylamide	QTOF	Statistical evaluation of glycoprofiles	[44]
EPO, human growth hormone	Pharmaceutical formulations	1 M acetic acid and 40 mM ammonium bicarbonate (pH 8.5)	No coating and linear polyacrylamide	hrTOF	Product characterization	[43]
EPO, model proteins	Aqueous solution, pharmaceutical formulation	0.5–2 M acetic acid	Several	QTOF	Coating comparison and optimization	[39]
Growth hormone, ins, hemoglobin	Aqueous solution	75 mM ammonium formate (pH 2.5 and 9.0) with ACN	No coating	TOF	Use of organic solvent to prevent protein adsorption	[40]
Human growth hormone, interferon-β-1a, oxytocin	Pharmaceutical preparation	Acetic acid (pH 3.0) and ammonium formate (pH 8.5)	PB-PVS and PB-DS-PB	TOF	Biopharmaceutical characterization	[35]
Ins	Pharmaceutical formulations	75 mM ammonium formate (pH 9.0) with 10% ACN	No coating	TOF	Product characterization	[45]
Ins b-chain, β-casein	Aqueous solution	10 mM ammonium bicarbonate-acetate (pH 7.0)	No coating	QIT	Online digestion	[61]
Polypeptides, small proteins	Cerebral spinal fluid	250 mM formic acid, 20% ACN	No coating	TOF	Biomarker discovery Alzheimer's disease	[60]
Polypeptides, small proteins	Human plasma	250 mM formic acid, 20% ACN	No coating	TOF	Biomarker discovery vascular disease	[59]
Polypeptides, small proteins	Human urine	250 mM formic acid, 20% ACN	No coating	TOF	Biomarker discovery various diseases	[46–57]
Polypeptides, small proteins	Human urine	250 mM formic acid, 20% ACN	No coating	TOF	Comparison LC-MS and CE-MS for biomarker analysis	[58]
Protein-drug conjugates	Aqueous solution	100 mM acetic acid (pH 3.1)	PEI	TOF	Product characterization	[23]
Protein-linker conjugates	Aqueous solution	100 mM acetic acid (pH 3.1)	PEI	TOF	Product characterization	[24]
α-1-Acid glycoprotein	Human serum	1 M acetic acid	DMA-EPyM	QTOF	Glycoprofiling	[37]
α-1-Acid glycoprotein	Human serum	1 M acetic acid	DMA-EPyM	QTOF	Statistical evaluation of glycoprofiles	[36]
Capillary isoelectric focusing						
BSA, myo, RNase, α-lac, β-LGA, β-LGB	Aqueous solution, rabbit serum	1% Beckman (pH 3–10) and 1% Ampholine (pH 4–6) mixture	No coating	SQ	Optimization for online cIEF-ESI-MS, Glycerol added to BGE to suppress EOF	[62]
CAII, myo, RNase A, β-LG	Aqueous solution	1–2% (v/v) Fluka pH 3–10 ampholytes	No coating and polyvinylalcohol	IT/TOF	Optimization for online cIEF-ESI-MS	[30]
BSA, myo, RNase A, β-LG, cell lysate	Aqueous solution, *E. coli* whole cell lysate	0.6% pharmalytes	Linear polyacrylamide	IT	cIEF-IMER-RPLC-MS for ampholyte removal and subsequent peptide analysis	[31]

a) CAII, carbonic anhydrase; cyt c, cytochrome c; lys, lysozyme; myo, myoglobin; RNase A, ribonuclease A; ubi, ubiquitin; α-CT, α-chymotrypsinogen; α-lac, α-lactalbumin; β-LG, β-lactoglobulin; β-LGA, β-lactoglobulin A; β-LGB, β-lactoglobulin B.

b) DMA-EPyM, *N,N*-dimethylacrylamide-ethylpyrrolidine methacrylate; PEI, polyethyleneimine; PVS, poly(vinylsulfonic acid).

c) hrTOF, high-resolution time-of-flight; IT, iontrap; QIT, quadrupole iontrap; SQ, single quadrupole.

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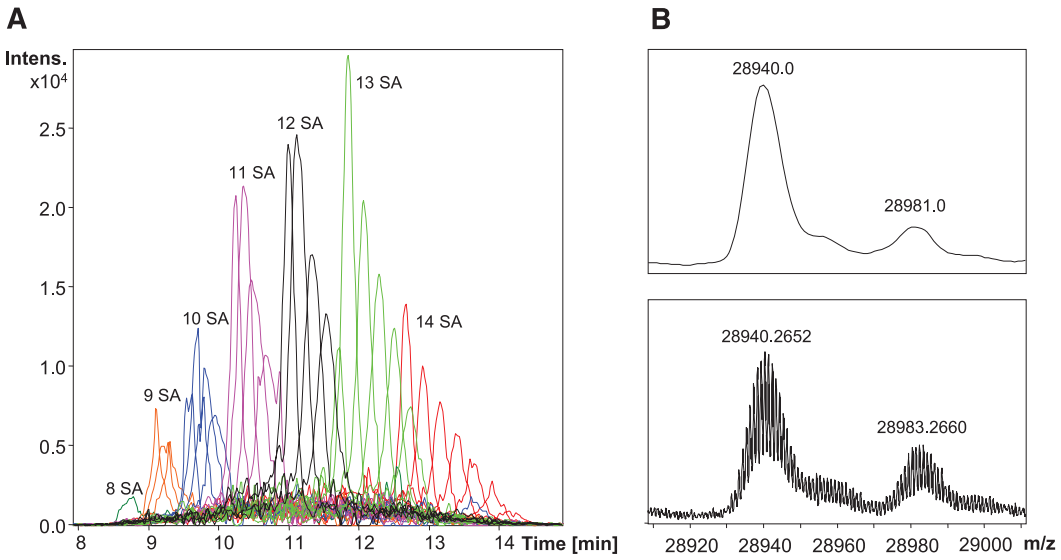


Figure 3. (A) Extracted ion electropherograms (EIEs) of an EPO analysis with CE-MS using a neutrally coated capillary and 1 M acetic acid as BGE. All traces are EIEs of the average mass of the most abundant charge state. Traces of same color represent EIEs of glycoforms showing the same number of sialic acids (SA). Within these groups the glycoforms are separated by the number of hexose-*N*-acetylhexosamine units (repeats). (B) Comparison of two deconvoluted mass spectra of a selected EPO glycoform. Top trace represents a spectrum obtained by a standard TOF-MS (R ≈ 10 000), whereas the bottom trace shows a deconvoluted spectrum obtained by a high-resolution TOF-MS (R ≈ 40 000). Reprinted from [43], with permission from Elsevier.

applying the PB-poly(vinylsulfonic acid) coating. Upon heat exposure of this protein, oxidation, sulfonate formation, and deamidation were observed. The peptide oxytocin and the glycoprotein recombinant human interferon-β-1a were analyzed with CE-MS at low pH using a PB-DS-PB coated capillary. Oxytocin samples exposed to heat and low pH showed strong deamidation, whereas at medium and high pH mainly dimer and trisulfide formation occurred. Analysis of interferon-β-1a, an *N*-glycosylated protein, revealed a cluster of resolved peaks comprising at least ten glycoforms differing merely in sialic acid and hexose *N*-acetylhexosamine composition.

CZE-ESI-MS was also used for the characterization of different drug-lysozyme conjugates by Haselberg et al. [23]. The conjugates consisted of a kinase inhibitor coupled to lysozyme via a noncovalent platinum(II)-based linker. The porous tip sprayer (see Section 2.1.1) was employed for sheathless CE-ESI-MS interfacing. A positively charged polyethylenimine capillary coating was used in combination with a low-pH BGE. Under these conditions, narrow symmetrical peaks for the various reaction products were obtained (Fig. 4A and B) demonstrating that conjugates remained stable during the CE analysis and subsequent ESI. Components observed in the drug-protein products were assigned based on their relative migration times and on molecular mass as obtained by TOF-MS, revealing the presence of unmodified protein and protein with up to two drug molecules (Fig. 4B). The authors also used this method to characterize a llama antibody used for cancer treatment that was modified with *N*-succinimidyl-S-acetylthioacetate (SATA) [24]. The protein was randomly modified with SATA at the lysine residues. With CE-MS the unmodified protein and the protein with one to eight SATA-modifications could be identified and quantified.

2.2.2 Bioanalysis

The combination of CZE with ESI-MS has also been used for the analysis of intact proteins in biological samples. Ongay et al. employed a positively charged coating in combination with a BGE of low pH to characterize intact α-1-acid glycoprotein (AGP) [37]. This glycoprotein is mainly produced by the liver and its plasma concentration is altered during inflammation. Upon CE-MS analysis, a large amount of isoforms differing in attached glycans were found. Due to the high number of possible sugar substitutions and other minor modifications no unequivocal carbohydrate composition could be elucidated. To provide the carbohydrate composition, the deglycosylated protein, and released glycans were also analyzed with CE-MS. Combining all the data allowed the characterization of more than 150 AGP isoforms, differing in both the amino acid sequence and the carbohydrates attached. This approach was used to analyze AGP samples from 16 individuals (eight healthy, eight bladder cancer) [36]. The analytical data were evaluated employing different statistical techniques like ANOVA, principal component analysis, linear discriminant analysis, and partial least squares-discriminant analysis. Statistical differences between the two groups were observed using CE-ESI-MS data for intact AGP isoforms. The differences between the groups could be assigned to higher abundance of

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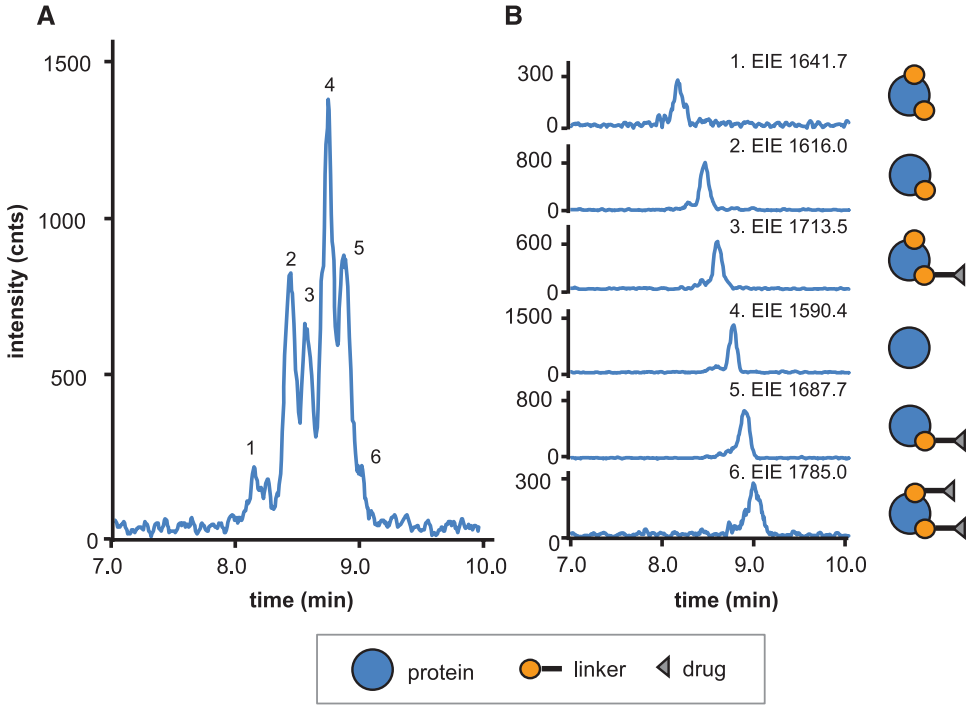


Figure 4. (A) Base-peak electropherogram obtained during CE-MS of a protein-drug conjugate using a positively charged coating in combination with a BGE of 100 mM acetic acid (pH 3.1). (B) EIEs for the main ion of peak 1–6 constructed at the indicated *m/z* values with interpretation of the observed species. Reprinted from [23], with permission from Elsevier.

AGP isoforms containing tri- and tetra-antennary fucosylated oligosaccharides in cancer patients.

The group of Mischak used CE-MS for the screening of human urine [46–58], human plasma [59], and human cerebrospinal fluid [60] in order to identify possible biomarkers. The authors focused on peptides and proteins with a molecular weight up to 20 kDa in their analysis. For all studies the same CE method employing a 250 mM formic acid BGE containing 20% ACN and a bare-fused silica capillary was used. 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 and healthy persons were compared. Differences between diseased and healthy indicated the presence of potential biomarkers, which were subsequently identified with CE-MS/MS and LC-MS/MS. In most cases peptide biomarkers resulted from the same source, like collagen, fibrinogen, albumin, antitrypsin, and amyloid-β. Apparently these are not disease specific. However, in some studies also unknown, and more specific, compounds were found that could become valuable biomarkers.

In a recent publication from the same group, a comparison between CE-TOF-MS and reversed phase LC-TOF-MS was made for bioanalysis using male and female urine samples [58]. Profiles obtained with CE-MS and LC-MS (Fig. 5A) were compared in terms of number of peptides, reproducibility, and mass range of detected peptides, reproducibility of peak area and migration time, and carry over. Both techniques had similar results in terms of number of peptides detected and the mass range over which the peptides were detected over. However, CE yielded higher coverage for the low- (<2000 Da) and high-molecular weight (>4000 Da) compounds, whereas LC provided more coverage in the 2000–4000 Da mass range (Fig. 5B). Other parameters did not yield any major differences.

2.2.3 Miscellaneous applications

Li et al. developed a 2D CE system that incorporates a replaceable enzymatic microreactor for on-line protein digestion [61]. In this system, trypsin was immobilized on magnetic beads, which were captured by a pair of magnets at the end of the first capillary. For analysis, proteins were separated in this first capillary. A fraction was then captured in the reactor for digestion. The formed peptides were periodically transferred to the second capillary for identification by CE-MS. Since the entire setup consists of three separate high-voltage supplies, a single stage could be controlled without interfering with the other stages. The suitability of the system was demonstrated by the separation and digestion of insulin chain b and α-casein. Protein separations were performed within 8 min, whereas each peptide fraction was analyzed within 1 min after also 1 min of digestion. In total, about 30 fractions were analyzed, leading up to 100% sequence coverage for the insulin chain b and 45% for α-casein within a total run time of 30 min.

Moini used sheathless CE-MS to study the stoichiometry of protein-metal complexes and metal displacement under native conditions [25]. Autoinducer inactivation enzyme A (AiiA) incubated with cobalt was used as a model for this purpose. It was analyzed using BGEs of pH 4.8 and pH 6.9 containing 0.1% PB as a dynamic coating. At pH 4.8 the

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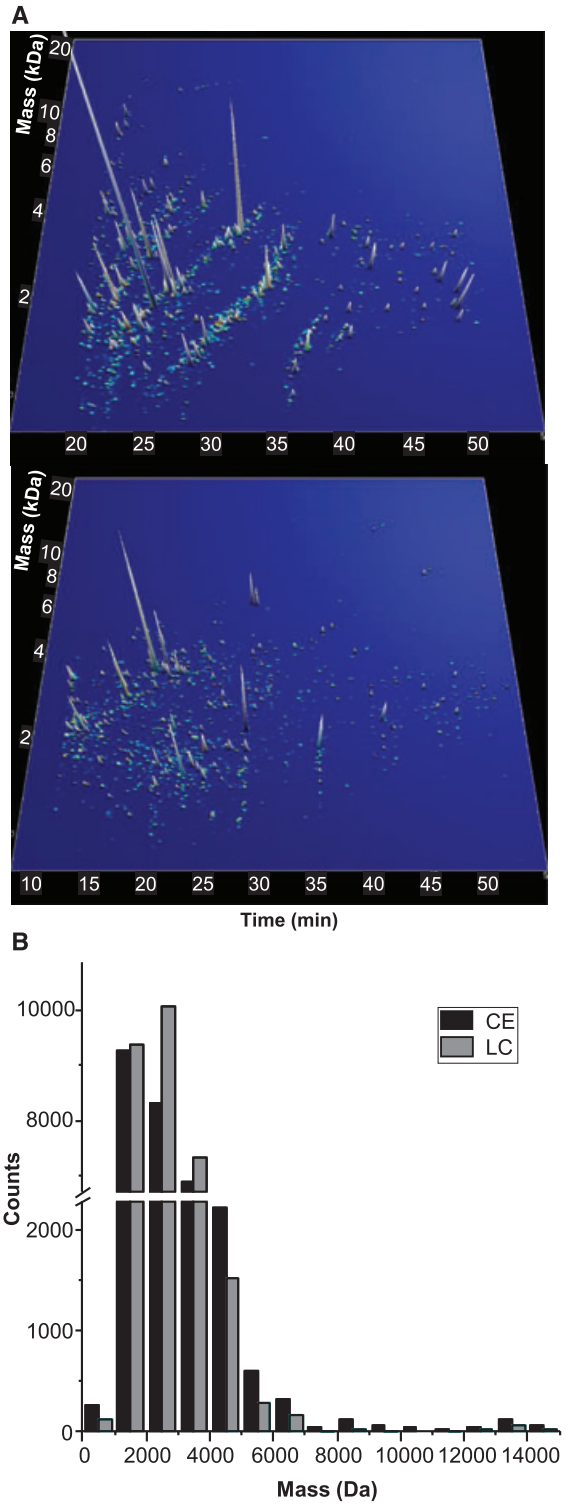


Figure 5. (A) Compiled peptide patterns of ten male urine samples run in CE-MS (top graph) and LC-MS systems (bottom graph). Normalized molecular mass (*y*-axis) is plotted against migration (CE) and retention time (LC). The mean signal intensity is represented in 3D depiction. (B) Histogram representing the mass ranges of the peaks detected in the CE-MS and LC-MS system [58]. Copyright Wiley-VCH Verlag GmbH & Co. Reproduced with permission.

protein-metal complex dissociated and only the protein was detected. In contrast, at pH 6.9 the protein-metal complex remained intact and the binding of two Co-ions to the protein was confirmed. Subsequently, AiiA-Co was incubated at various concentration of cadmium and the samples were analyzed with CE-MS. Loosely attached and nonspecific metal ions present in the sample solution were separated from the protein-metal complex prior to detection. Consequently, interferences of salts or extra metal ions that could hamper the accurate determination of stoichiometry were minimized. The results indicated that Co binds more tightly to AiiA than Cd; at a 10 times molar excess of Cd merely one Co ion is replaced, whereas a full replacement is reached at a 100 times molar excess of Cd.

Lecoeur et al. used a method previously developed in their laboratories for the on-line coupling of CIEF with ESI-MS [62]. The method comprised a discontinuous filling of the capillary with a volatile catholyte (60% of capillary volume) and a sample-ampholyte mixture (40% of capillary volume). They used this method to analyze milk whey proteins. To desorb these rather hydrophobic proteins from the inner capillary wall and to avoid capillary blockages, a new rinsing procedure compatible with MS detection was developed. In this procedure, the hydrochloric acid washing solution was replaced by a multi-step sequence based on the use of TFA, ammonia, and ethanol. Moreover, CIEF parameters like carrier ampholyte nature, capillary partial filling length with ampholyte/protein mixture and focusing time were optimized with respect to total analysis time, sensitivity, and precision on p*I* determination. Good separations of model proteins with low-nM LODs were achieved under optimized conditions. The developed method appeared to be suitable for the analysis of whey proteins in serum, although only preliminary data were provided.

3 CE-MALDI-MS

Over the past two years, few publications on CE-MALDI-MS have been published (Table 2). Lu et al. developed a novel strategy in which they coupled CGE with MS detection [63]. CGE is an established technique in the biopharmaceutical industry, especially for the characterization of monoclonal antibodies, and its coupling to MS detection has an added value for protein identification. The authors make use of a poly(tetrafluoroethylene) membrane placed in a grounded outlet reservoir that is moved during the separation (Fig. 6A). This way, separated proteins were collected at different places on the membrane (i.e. comparable to a common slab gel experiment). The authors demonstrated that they were able to wash off the SDS bound to the collected proteins and identify these proteins on-membrane with MALDI-TOF-MS. For now, the research focused on the analysis of model proteins. The limiting factor was found to be the separation efficiency of CGE for proteins of similar molecular weight. However, proteins that differ significantly in

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Table 2. CE-MS for intact protein analysis using MALDI or ICP interfaces

Analytes	Sample matrix	CE mode	BGE/ampholytes	Capillary coating	MALDI matrix/elements	Remarks	Ref.
CE-MALDI–MS							
Model proteins, apolipoprotein A-I	Aqueous solution, *E. coli* extract	CIEF	Pharmalyte, 2% CHAPS and 0.5 mM ammonia acetate	Cross-linked polyacrylamide	α-Cyano-4-hydroxycinnamic acid	Optimization spotting conditions	[65]
Model proteins	Aqueous solution	CGE	4% acrylamide in 60 mM Tricine – 21 mM Tris (pH 7.6) and 0.25% SDS	Cross-linked polyacrylamide	α-Cyano-4-hydroxycinnamic acid	Coupling CGE with MALDI through membrane collector	[63]
Model proteins	Aqueous solution	CZE	83.3 mM ammonium acetate (pH 4.0)	Hydroxypropyl cellulose	Sinapinic acid	Field-enhanced sample injection preconcentration	[64]
CE-ICP–MS							
Human serum albumin	Aqueous solution, human urine	CZE	20 mM Tris–ammonium acetate (pH 8.0)	No coating	Au	Sample incubation with Au nanoparticles for enhanced sensitivity	[68]
Human serum albumin	Aqueous solution	CZE	10 mM ammonium acetate	No coating	Sn	ACE experiments to determine strength of compound-protein interaction	[66]
Human serum albumin, transferrin	Mouse plasma	CZE	50 mM formic acid	No coating	S, Ru	Comparison with SEC-ICP-MS	[67]

Note: In all cases, either a TOF mass spectrometer (CE-MALDI-MS) or a single quadruple mass analyzer (CE-ICP-MS) was used for detection.

Figure 6. (A) Schematic diagram of the SDS-CGE setup with membrane collector. (B) Mass spectra obtained on different positions (correlating to different migration times) on the membrane. Lysozyme (14 kDa) is visible in the green spectra. Trypsinogen (24 kDa) appears primarily in the blue traces. AcrA (42 kDA) signals show up in the red three traces. Phosphorylase b (97 kDa) was not detectable under the used experimental conditions. Reprinted with permission from [63]. Copyright 2011 American Chemical Society.

molecular weight could be nicely separated and detected with CGE-MALDI-MS (Fig. 6B).

In order to increase the sensitivity of CZE-MALDI-MS, Pourhaghighi et al. developed a field-enhanced sample injection strategy for intact proteins [64]. Injections of intact proteins were performed electrokinetically for 8 min, which resulted in an increase of peak area between a factor 1000 and 8000 compared to normal electrokinetic injections. In spite of the use of a high conductivity buffer required to perform successful field-enhanced sample injection preconcentration, hampering coupling with ESI-MS, the developed methodology could be efficiently used in conjunction with MALDI-MS. The high-resolution separation and sensitive detection enabled identification of low-nM concentrations of different proteins.

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Cheng et al. focused their research within the field of CIEF-MALDI-MS for intact proteins on the optimization of spotting conditions [65]. They first placed a drop of water onto a MALDI-MS target. Then a fraction of the CIEF-separated sample was spotted at the center region close to the bottom of the droplet. Because the small additive molecules (like carrier ampholytes) diffuse faster than proteins, relatively more protein molecules remained in the center region of the sample spot after solvent evaporation. By directing the laser to this region to ablate the sample, they could improve the S/N ratio with a factor 2–10. Their approach was applied for the analysis of model proteins and apolipoprotein A–I, a membrane protein expressed in *E. coli*.

4 CE-ICP-MS

CE-ICP-MS is limited to metal-containing and metal-binding proteins, but for this type of proteins highly selective information can be obtained. In all reports on CE-ICP-MS over the past two years (Table 2) the binding of metal-containing compounds to HSA was studied. Sun et al. used ACE and nonequilibrium capillary electrophoresis assays of equilibrium mixtures combined with ICP-MS as a tool to determine the binding constants of organotin compounds toward HSA and to compare between the two affinity methods [66]. CE-ICP-MS allowed the use of nonvolatile BGEs, such as Tris, resembling physiological conditions during separation, while still obtaining good sensitivity. Notably, such conditions might not feasible in CE-ESI-MS. With both affinity approaches binding constants (log K_b) could be easily derived. Using ACE these were on average around 6.0 for four different organotin components, which was in agreement with other reports on this interaction. For nonequilibrium capillary electrophoresis assays of equilibrium mixtures, binding constants were around 7.0 (one order of magnitude higher). Although the authors did not fully investigate the reason for the difference, they stated that it could be due to dissociation of the complex during CE or HSA adsorption to the inner wall of the capillary, where the consequent peak tailing affected the mathematical analysis of the peak areas.

Bytzek et al. used CE-ICP-MS to monitor the binding of ruthenium–indazole complexes to serum proteins [67]. The complexes studied are promising anticancer agents undergoing clinical trials and serum proteins are their first available biological-binding partners. It was found that the ruthenium–indazole complexes mainly interact with HSA and its dimer. These two species could be separated by the use of a positively charged PB coating in combination with a low-pH BGE. Next to CE-ICP-MS, the authors also performed 1D (size exclusion chromatography) and 2D (size exclusion chromatography—ion chromatography) LC separations to determine the molar-binding ratio of Ru to the protein. In general, the observed molar ratio was slightly higher with CE compared to the LC approaches. It was concluded that the LC-based methods were more sensitive, whereas the CE approach was considerably quicker.

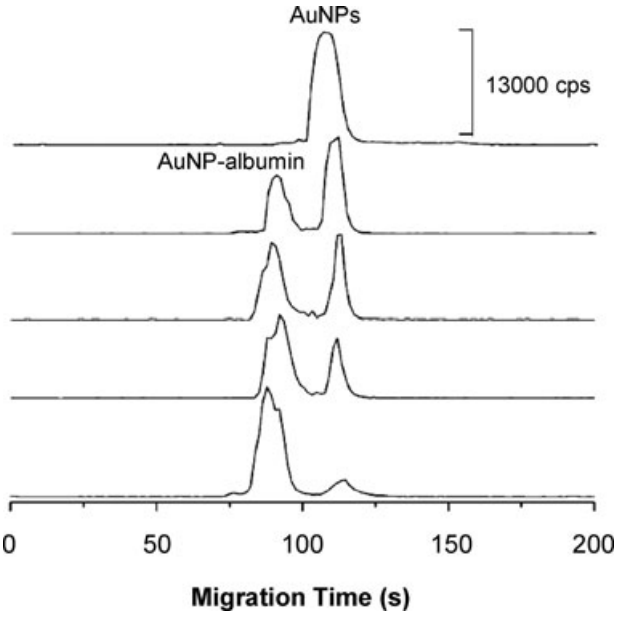


Figure 7. Electropherograms obtained by CE-ICP-MS for 6 pM AuNPs incubated with albumin concentration increased from top to bottom: 0, 100, 150, 200, 250 pM. The traces represent the signal of the ^{197}Au isotope. Reprinted with permission from [68]. Copyright 2010 American Chemical Society.

In CE and CE-MS the limited sensitivity is an important issue. For CE-ICP-MS Liu et al. developed a strategy to improve the quantification of human urinary proteins [68]. In their approach, urine samples were incubated with gold nanoparticles (AuNPs) to form protein-AuNPs adducts. The authors demonstrated that, after optimization, this strategy is selective for HSA, where the protein and AuNPs bind due to electrostatic interactions. The albumin-AuNP adduct was separated from the unbound AuNPs by CE using a BGE of ammonium acetate and Tris (pH 8.0) (Fig. 7). As a result of AuNPs-tagging, on average more than 2000 gold atoms were attached to each albumin molecule to successfully achieve a significant amplification of ICP-MS signal. An LOD for HSA of 0.5 pM was obtained, which was at least 1000 times more sensitive than other separation-detection schemes.

5 Conclusions

The ongoing development and applicability of CE-MS for qualitative and quantitative protein studies in various fields was demonstrated in almost 40 publications over the past two years. From the number of publications it is obvious that CE-ESI-MS is the most widely used and mature combination. It should be noted that, although most studies were application driven, still methodological studies are performed to push the technique forward. Especially the new interfacing strategies seem to open new avenues for intact protein analysis, outperforming common sheath-liquid interfacing in terms of sensitivity. However, whether these interfaces also

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can provide similar or improved long-term reproducibility as sheath liquid interfaces remains to be investigated. The coupling of CE with optical detection and mass spectrometric detection could be an interesting way to improve information content. CE-MS hyphenated with laser-induced fluorescence [69] or wavelength-resolved fluorescence [70] could provide a good platform for selective quantitative detection and identification in a single setup.

From an application point of view, it seems the CE-ESI-MS has found its main use in the characterization of (glycosylated) biopharmaceuticals and the analysis of proteins in biological samples. Especially, the first application area has gained increasing attention since the pharmaceutical industry is moving more and more in this direction. Since the main biopharmaceuticals on the market are antibody based, it is foreseen that research will be more directed to this field. However, due to their high molecular weight and significant heterogeneity the analysis of antibodies will not be straightforward. Modifications on antibodies (e.g. deamidations or glycosylation) will have a relatively small effect on the overall charge and size of the 150 kDa protein. Therefore, the separation of these isoforms will be challenging, most likely requiring low-EOF conditions. Creating gas phase ions by ESI from such high-molecular weight compounds after a CE separation will require thorough optimization and characterization of separation and ionization parameters. Especially for these large molecules, the use of mass spectrometers with a high resolution should be considered. Orbitrap or Fourier transform ion cyclotron resonance mass analyzers, which provide mass resolution of several 100 000, would be very suitable for this task. The long duty cycles required to obtain this high resolution may compromise the analysis of narrow CE peaks, whereas shorter cycles may lead to reduced sensitivity. 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, for example, wavelength resolved fluorescence [71] seems very useful.

For both CE-MALDI-MS and CE-ICP-MS also a state of maturity is reached and people are now focusing more on the applicability of the techniques. The most interesting methodological development is the coupling of CGE to MALDI-MS. The former is a standard method in biopharmaceutical industry for the quality control of proteins. Now, with the added mass spectrometric dimension, more detailed information about proteins can be obtained. However, it will take some effort to make this into a routine analysis. The coupling of CGE or CIEF with ICP-MS could also be interesting option, which will definitely increase the applicability of CE-ICP-MS toward, for example, the analysis of protein phosphorylation and heme-containing proteins.

Overall, 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. Especially compared to current LC-MS methods, CE-MS is strong in cases where separation of highly similar protein species or complex mixtures of proteins is paramount (like glycoform discrimination). Development of novel stationary phases for LC separations of intact proteins is ongoing, so it is foreseen that LC-MS will also find more application in this field.

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6 References

[1] Ramautar, R., Heemskerk, A. A. M., Hensbergen, P. J., Deelder, A. M., Busnel, J. M., Mayboroda, O. A., *J. Proteomics* 2012, *75*, 3814–3828.

[2] Ramautar, R., Mayboroda, O. A., Somsen, G. W., de Jong, G. J., *Electrophoresis* 2011, *32*, 52–65.

[3] Rittgen, J., Pütz, M., Zimmermann, R., *Electrophoresis* 2012, *33*, 1595–1605.

[4] Suntornsuk, L., *Anal. Bioanal. Chem.* 2010, *398*, 29–52.

[5] Castro-Puyana, M., García-Cañas, V., Simó, C., Cifuentes, A., *Electrophoresis* 2012, *33*, 147–167.

[6] Haselberg, R., de Jong, G. J., Somsen, G. W., *J. Chromatogr. A* 2007, *1159*, 81–109.

[7] Haselberg, R., de Jong, G. J., Somsen, G. W., *Electrophoresis* 2011, *32*, 66–82.

[8] Staub, A., Schappler, J., Rudaz, S., Veuthey, J. L., *Electrophoresis* 2009, *30*, 1610–1623.

[9] Wysocki, V. H., Resing, K. A., Zhang, Q., Cheng, G., *Methods* 2005, *35*, 211–222.

[10] Ferguson, P. L., Kuprowski, M. C., Boys, B. L., Wilson, D. J., Pan, J., Konermann, L., *Curr. Anal. Chem.* 2009, *5*, 186–204.

[11] Hommerson, P., Khan, A. M., de Jong, G. J., Somsen, G. W., *Mass Spectrom. Rev.* 2011, *30*, 1096–1120.

[12] Stutz, H., *Electrophoresis* 2005, *26*, 1254–1290.

[13] Kannamkumarath, S. S., Wrobel, K., Wrobel, K., B'Hymer, C., Caruso, J. A., *J. Chromatogr. A* 2002, *975*, 245–266.

[14] Haselberg, R., Ratnayake, C. K., de Jong, G. J., Somsen, G. W., *J. Chromatogr. A* 2010, *1217*, 7605–7611.

[15] Zamfir, A. D., *J. Chromatogr. A* 2007, *1159*, 2–13.

[16] Monton, M. R. N., Terabe, S., *Anal. Sci.* 2005, *21*, 5–13.

[17] Moini, M., *Anal. Chem.* 2007, *79*, 4241–4246.

[18] Maxwell, E. J., Zhong, X., Zhang, H., Van Zeijl, N., Chen, D. D. Y., *Electrophoresis* 2010, *31*, 1130–1137.

[19] Ramautar, R., Busnel, J. M., Deelder, A. M., Mayboroda, O. A., *Anal. Chem.* 2012, *84*, 885–892.

[20] Heemskerk, A. A. M., Busnel, J. M., Schoenmaker, B., Derks, R. J. E., Klychnikov, O., Hensbergen, P. J.,

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Deelder, A. M., Mayboroda, O. A., *Anal. Chem.* 2012, *84*, 4552–4559.

[21] Faserl, K., Sarg, B., Kremser, L., Lindner, H., *Anal. Chem.* 2011, *83*, 7297–7305.

[22] Bonvin, G., Veuthey, J. L., Rudaz, S., Schappler, J., *Electrophoresis* 2012, *33*, 552–562.

[23] Haselberg, R., Harmsen, S., Dolman, M. E. M., de Jong, G. J., Kok, R. J., Somsen, G. W., *Anal. Chim. Acta* 2011, *698*, 77–83.

[24] van der Meel, R., Oliveira, S., Altintas, I., Haselberg, R., van der Veeken, J., Roovers, R. C., van Bergen en Henegouwen, P. M. P., Storm, G., Hennink, W. E., Schiffelers, R. M., Kok, R. J., *J. Control Release* 2012, *159*, 281–289.

[25] Moini, M., *Rapid Commun. Mass Spectrom.* 2010, *24*, 2730–2734.

[26] Maxwell, E. J., Ratnayake, C., Jayo, R., Zhong, X., Chen, D. D. Y., *Electrophoresis* 2011, *32*, 2161–2166.

[27] Maxwell, E. J., Zhong, X., Chen, D. D. Y., *Anal. Chem.* 2010, *82*, 8377–8381.

[28] Zhao, S. S., Zhong, X., Chen, D. D. Y., *Electrophoresis* 2012, *33*, 1322–1330.

[29] Zhong, X., Maxwell, E. J., Chen, D. D. Y., *Anal. Chem.* 2011, *83*, 4916–4923.

[30] Zhong, X., Maxwell, E. J., Ratnayake, C., Mack, S., Chen, D. D. Y., *Anal. Chem.* 2011, *83*, 8748–8755.

[31] Wang, T., Ma, J., Wu, S., Yuan, H., Zhang, L., Liang, Z., Zhang, Y., *Electrophoresis* 2011, *32*, 2848–2856.

[32] Stutz, H., *Electrophoresis* 2009, *30*, 2032–2061.

[33] Lucy, C. A., MacDonald, A. M., Gulcev, M. D., *J. Chromatogr. A* 2008, *1184*, 81–105.

[34] Haselberg, R., de Jong, G. J., Somsen, G. W., *Anal. Chim. Acta* 2010, *678*, 128–134.

[35] Haselberg, R., Brinks, V., Hawe, A., de Jong, G. J., Somsen, G. W., *Anal. Bioanal. Chem.* 2011, *400*, 295–303.

[36] Ongay, S., Martín-Álvarez, P. J., Neusüß, C., de Frutos, M., *Electrophoresis* 2010, *31*, 3314–3325.

[37] Ongay, S., Neusüß, C., *Anal. Bioanal. Chem.* 2010, *398*, 845–855.

[38] Huhn, C., Ramautar, R., Wuhrer, M., Somsen, G. W., *Anal. Bioanal. Chem.* 2010, *396*, 297–314.

[39] Taichrib, A., Pioch, M., Neusüß, C., *Electrophoresis* 2012, *33*, 1356–1366.

[40] Staub, A., Comte, S., Rudaz, S., Veuthey, J. L., Schappler, J., *Electrophoresis* 2010, *31*, 3326–3333.

[41] Tran, N. T., Ayed, I., Pallandre, A., Taverna, M., *Electrophoresis* 2010, *31*, 147–173.

[42] Sikanen, T., Aura, S., Franssila, S., Kotiaho, T., Kostiainen, R., *Anal. Chim. Acta* 2012, *711*, 69–76.

[43] Taichrib, A., Pelzing, M., Pellegrino, C., Rossi, M., Neusüß, C., *J. Proteomics* 2011, *74*, 958–966.

[44] Taichrib, A., Pioch, M., Neusüß, C., *Anal. Bioanal. Chem.* 2012, *403*, 797–805.

[45] Staub, A., Rudaz, S., Veuthey, J. L., Schappler, J., *J. Chromatogr. A* 2010, *1217*, 8041–8047.

[46] Alkhalaff, A., Zürbig, P., Bakker, S. J. L., Bilo, H. J. G., Cerna, M., Fischer, C., Fuchs, S., Janssen, B., Medek, K., Mischak, H., Roob, J. M., Rossing, K., Rossing, P., Rychlík, I., Sourij, H., Tiran, B., Winklhofer-Roob, B. M., Navis, G. J., *PLoS ONE* 2010, *5*, e13421.

[47] Andersen, S., Mischak, H., Zürbig, P., Parving, H. H., Rossing, P., *BMC Nephrol.* 2010, *11*, 29.

[48] Bandin, F., Siwy, J., Breuil, B., Mischak, H., Bascands, J. L., Decramer, S., Schanstra, J. P., *J. Urology* 2012, *187*, 1006–1011.

[49] Carty, D. M., Siwy, J., Brennand, J. E., Zürbig, P., Mullen, W., Franke, J., McCulloch, J. W., North, R. A., Chappell, L. C., Mischak, H., Poston, L., Dominiczak, A. F., Delles, C., *Hypertension* 2011, *57*, 561–569.

[50] Dakna, M., Harris, K., Kalousis, A., Carpentier, S., Kolch, W., Schanstra, J. P., Haubitz, M., Vlahou, A., Mischak, H., Girolami, M., *BMC Bioinformatics* 2010, *11*, 594.

[51] Delles, C., Schiffer, E., Von Zur Muhlen, C., Peter, K., Rossing, P., Parving, H. H., Dymott, J. A., Neisius, U., Zimmerli, L. U., Snell-Bergeon, J. K., Maahs, D. M., Schmieder, R. E., Mischak, H., Dominiczak, A. F., *J. Hypertens.* 2010, *28*, 2316–2322.

[52] Good, D. M., Zürbig, P., Argilés, À., Bauer, H. W., Behrens, G., Coon, J. J., Dakna, M., Decramer, S., Delles, C., Dominiczak, A. F., Ehrich, J. H. H., Eitner, F., Fliser, D., Frommberger, M., Ganser, A., Girolami, M. A., Golovko, I., Gwinner, W., Haubitz, M., Herget-Rosenthal, S., Jankowski, J., Jahn, H., Jerums, G., Julian, B. A., Kellmann, M., Kliem, V., Kolch, W., Krolewski, A. S., Luppi, M., Massy, Z., Melter, M., Neusüß, C., Novak, J., Peter, K., Rossing, K., Rupprecht, H., Schanstra, J. P., Schiffer, E., Stolzenburg, J. U., Tarnow, L., Theodorescu, D., Thongboonkerd, V., Vanholder, R., Weissinger, E. M., Mischak, H., Schmitt-Kopplinr, P., *Mol. Cell. Proteomics* 2010, *9*, 2424–2437.

[53] Kistler, A. D., Siwy, J., Breunig, F., Jeevaratnam, P., Scherl, A., Mullen, W., Warnock, D. G., Wanner, C., Hughes, D. A., Mischak, H., Wüthrich, R. P., Serra, A. L., *PLoS ONE* 2011, *6*, e20534.

[54] Lopez, L. M., Mullen, W., Zürbig, P., Harris, S. E., Gow, A. J., Starr, J. M., Porteous, D. J., Mischak, H., Deary, I. J., *Intelligence* 2011, *39*, 46–53.

[55] Maahs, D. M., Siwy, J., Argilés, A., Cerna, M., Delles, C., Dominiczak, A. F., Gayrard, N., Iphöfer, A., Jänsch, L., Jerums, G., Medek, K., Mischak, H., Navis, G. J., Roob, J. M., Rossing, K., Rossing, P., Rychlík, I., Schiffer, E., Schmieder, R. E., Wascher, T. C., Winklhofer-Roob, B. M., Zimmerli, L. U., Zürbig, P., Snell-Bergeon, J. K., *PLoS ONE* 2010, *5*, e13051.

[56] Metzger, J., Chatzikyrkou, C., Broecker, V., Schiffer, E., Jaensch, L., Iphoefer, A., Mengel, M., Mullen, W., Mischak, H., Haller, H., Gwinner, W., *Proteomics Clin. Appl.* 2011, *5*, 322–333.

[57] Mullen, W., Gonzalez, J., Siwy, J., Franke, J., Sattar, N., Mullan, A., Roberts, S., Delles, C., Mischak, H., Albalat, A., *J. Agr. Food Chem.* 2011, *59*, 12850–12857.

[58] Mullen, W., Albalat, A., Gonzalez, J., Zerefos, P., Siwy, J., Franke, J., Mischak, H., *Electrophoresis* 2012, *33*, 567–574.

[59] Schiffer, E., Liabeuf, S., Lacroix, C., Temmar, M., Renard, C., Monsarrat, B., Choukroun, G., Lemke, H. D., Vanholder, R., Mischak, H., Massy, Z. A., *J. Hypertens.* 2011, *29*, 783–790.

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[60] Jahn, H., Wittke, S., Zürbig, P., Raedler, T. J., Arlt, S., Kellmann, M., Mullen, W., Eichenlaub, M., Mischak, H., Wiedemann, K., *PLoS ONE* 2011, *6*, e26540.

[61] Li, Y., Wojcik, R., Dovichi, N. J., *J. Chromatogr. A* 2011, *1218*, 2007–2011.

[62] Lecoeur, M., Gareil, P., Varenne, A., *J. Chromatogr. A* 2010, *1217*, 7293–7301.

[63] Lu, J. J., Zhu, Z., Wang, W., Liu, S., *Anal. Chem.* 2011, *83*, 1784–1790.

[64] Pourhaghighi, M. R., Busnel, J. M., Girault, H. H., *Electrophoresis* 2011, *32*, 1795–1803.

[65] Cheng, C., Lu, J. J., Wang, X., Roberts, J., Liu, S., *Electrophoresis* 2010, *31*, 2614–2621.

[66] Sun, J., He, B., Liu, Q., Ruan, T., Jiang, G., *Talanta* 2012, *93*, 239–244.

[67] Bytzek, A. K., Boeck, K., Hermann, G., Hann, S., Keppler, B. K., Hartinger, C. G., Koellensperger, G., *Metallomics* 2011, *3*, 1049–1055.

[68] Liu, J. M., Li, Y., Jiang, Y., Yan, X. P., *J. Proteome Res.* 2010, *9*, 3545–3550.

[69] Huhn, C., Ruhaak, L. R., Mannhardt, J., Wuhrer, M., Neusüß, C., Deelder, A. M., Meyer, H., *Electrophoresis* 2012, *33*, 563–566.

[70] De Kort, B. J., De Jong, G. J., Somsen, G. W., *Electrophoresis* 2010, *31*, 2861–2868.

[71] De Kort, B. J., Ten Kate, G. A., De Jong, G. J., Somsen, G. W., *Anal. Chem.* 2011, *83*, 6060–6067.

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