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Review

Capillary electrophoresis–mass spectrometry for the analysis of intact proteins 2007–2010

CE coupled to 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 CIEF with MS and chip-based CE-MS are treated. Furthermore, CE-MS of intact proteins involving ESI, MALDI and ICP ionization 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.

Keywords:
CE / ESI / ICP ionization / Intact proteins / MALDI

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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. In addition, to increase the reliability of (selective) detection, a preceding separation can be critical.

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Abbreviations: EIE, extracted-ion electropherogram; **FACE,** frontal analysis CE; **FT-ICR,** Fourier transform ion cyclotron resonance; **hGH,** human growth hormone; **RPLC,** reversed-phase liquid chromatography; **Tf,** transferrin; **TIE,** total-ion electropherogram

Next to more conventional analytical techniques, such as liquid chromatography, size-exclusion chromatography and gel electrophoresis [1], 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 post-translational modifications like glycosylation or phosphorylation can potentially change the electrophoretic mobility of a protein, and can therefore be differentiated with CE. 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

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detection technique for CE. Over the years, various interfaces for several types of ionization sources have been developed. For CE-MS of proteins, ESI, MALDI and ICP ionization 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 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, such as *e.g.* glycosylation [6] and oxidation [7], can be determined. Furthermore, information on the conformation of proteins (native *versus* denatured) can also be 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. Owing 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 into 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 with 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 (Fig. 1A) [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 EOF. Three model proteins were separated within 14 min using a

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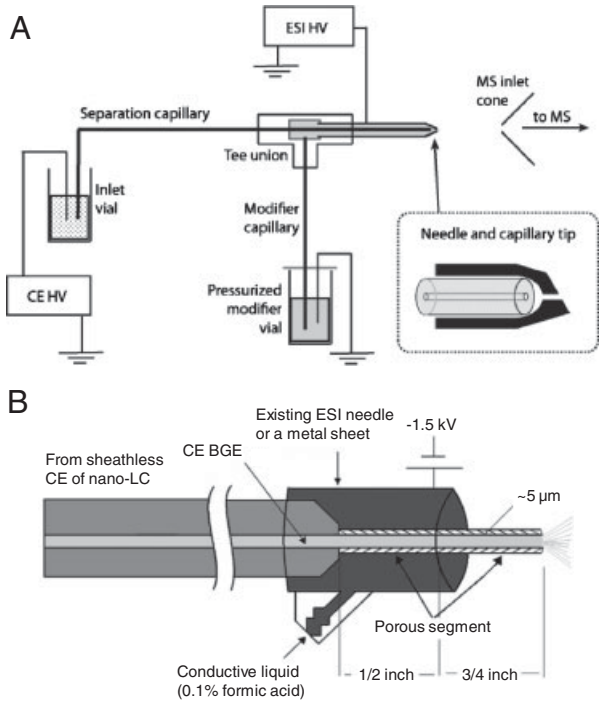


Figure 1. Schematic illustration of (A) the interface introduced by Maxwell *et al.* [19] (Copyright Wiley-VCH Verlag GmbH & Co. Reproduced with permission), including a dissected view of needle tip with inserted capillary in the inset and (B) the interface with a porous tip capillary positioned in a stainless steel capillary as described by Moini (Reprinted with permission from [20]. Copyright 2007 American Chemical Society).

neutral coated capillary and an acidic BGE, and good quality mass spectra 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 (Fig. 1B). 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 of 50 to 140 compared with 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). Owing 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.

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 1, 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 presented 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.

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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						
Antithrombin	Aqueous solution	30 mM ammonium hydrogencarbonate (pH 8.5)	No coating	IT	Frontal analysis to measure protein–ligand interac tion	[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 acid, 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% ACN	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% ACN	No coating	TOF/FT-ICR	Biomarker discovery cancer	[46, 56]
Polypeptides, small proteins	Human urine	250 mM formic acid, 20% ACN	No coating	TOF	Biomarker discovery vasculitis	[47]
Polypeptides, small proteins	Human urine	250 mM formic acid, 20% ACN	No coating	TOF	Biomarker discovery coronary artery diseases	[48, 49]
Polypeptides, small proteins	Human urine	250 mM formic acid, 20% ACN	No coating	TOF	Biomarker discovery kidney diseases	[50, 51, 55]
Polypeptides, small proteins	Human urine	250 mM formic acid, 20% ACN	No coating	TOF	Biomarker discovery lithium-induced nephratology	[52]
Polypeptides, small proteins	Human urine	250 mM formic acid, 20% ACN	No coating	TOF	Proteomic patterning graft-versus-host disease	[53]
Polypeptides, small proteins	Human urine	250 mM formic acid, 20% ACN	No coating	TOF	Biomarker discovery various diseases	[54]
Polypeptides, small proteins	Rat urine	250 mM formic acid, 20% ACN	No coating	TOF/FT-ICR	Peptidomic analysis; comparing different MS analyzers	[57]
Polypeptides, small proteins	Rat urine	250 mM formic acid, 20% ACN	No coating	TOF	Proteomic patterning drug-induced nephrotoxicity	[58]

Table 1. Continued

Analytes[a)]	Matrix	BGE/ampholytes	Capillary coating[b)]	Mass analyzer[c)]	Remarks	Ref.
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	ACN/isopropanol/formic acid/water (40/20/2/38)	EPyM/DMA	TOF/IT	Comparing mass analyzers for intact protein detection	[60]
Zein-proteins	Maize extracts	ACN/isopropanol/formic acid/water (40/20/2/38)	EPyM/DMA	IT	Comparing strains of maize	[59]
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 acid (50/50)	PolyE-323	QTOF	Characterizing chip CE-MS	[33]
Hb	Single cells	Methanol/2% acetic acid (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]

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

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 (vinyl sulfonic 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.

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2.1.3 CIEF

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 are 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 (Fig. 2A–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 (Fig. 2D). 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 an 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 polypeptides were detected in a sequence that lasted less than 8 h. The p*I versus* Mw 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] (Fig. 3A and B). 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 (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 (Fig. 3C). In a

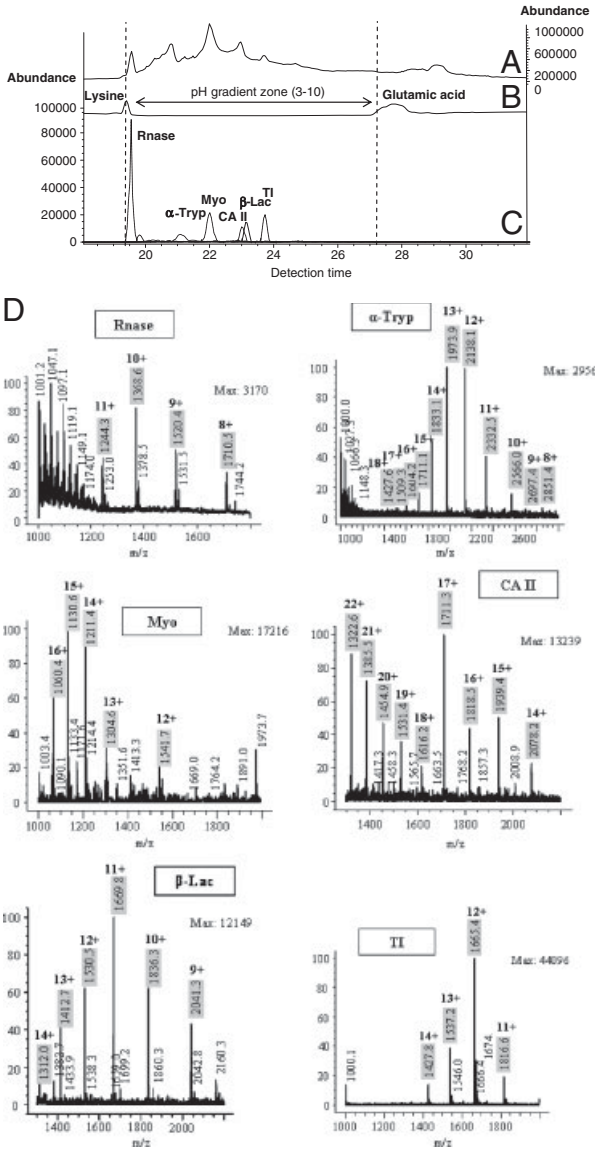


Figure 2. (A) Total-ion electropherogram (TIE) of the CIEF-ESI-MS analysis of six intact proteins. (B) extracted-ion electropherogram at *m/z* 148 to visualize lysine and glutamic acid, which indicate the beginning and end of the pH gradient. (C) Extracted-ion electropherograms of ribonuclease (Rnase), 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]. Copyright Wiley-VCH Verlag GmbH & Co. Reproduced with permission.

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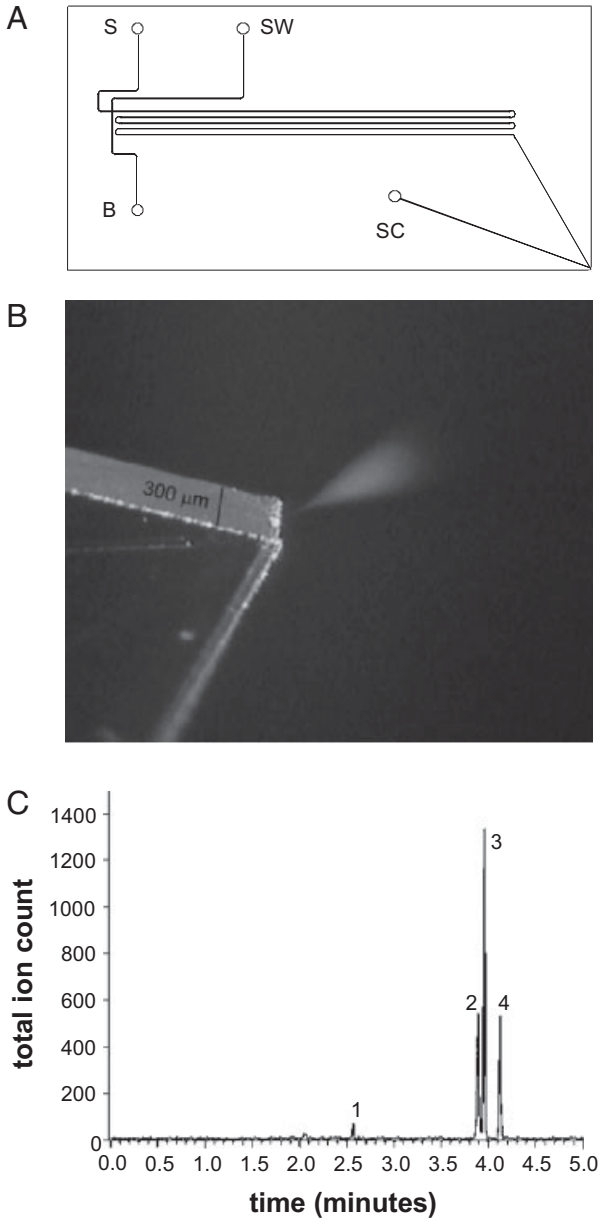


Figure 3. (A) Schematic representation of a CE-ESI-MS chip design for intact protein analysis. The length of the separation channel was 20.5 cm from the injection cross to the outlet. The reservoirs are labeled as S (sample), B (buffer), SW (sample waste) and SC (side channel). (B) Image of the electrospray 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. Reprinted with permission from [33]. Copyright 2008 American Chemical Society.

following publication, the usefulness of this chip CE-MS setup 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 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 1. In the following paragraphs some typical examples are outlined, focusing on glycoproteins, biopharmaceuticals, protein–ligand complexes, biomarkers and food 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]. $VEGF_{165}$ 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* (Fig. 4A). 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 (Fig. 4B). After deconvolution of the mass spectra, 16 glycoforms of intact $VEGF_{165}$ were identified (Fig. 4C). The glycosylation of this protein is mainly based on biantennary structures consisting of hexose (Hex) and *N*-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.

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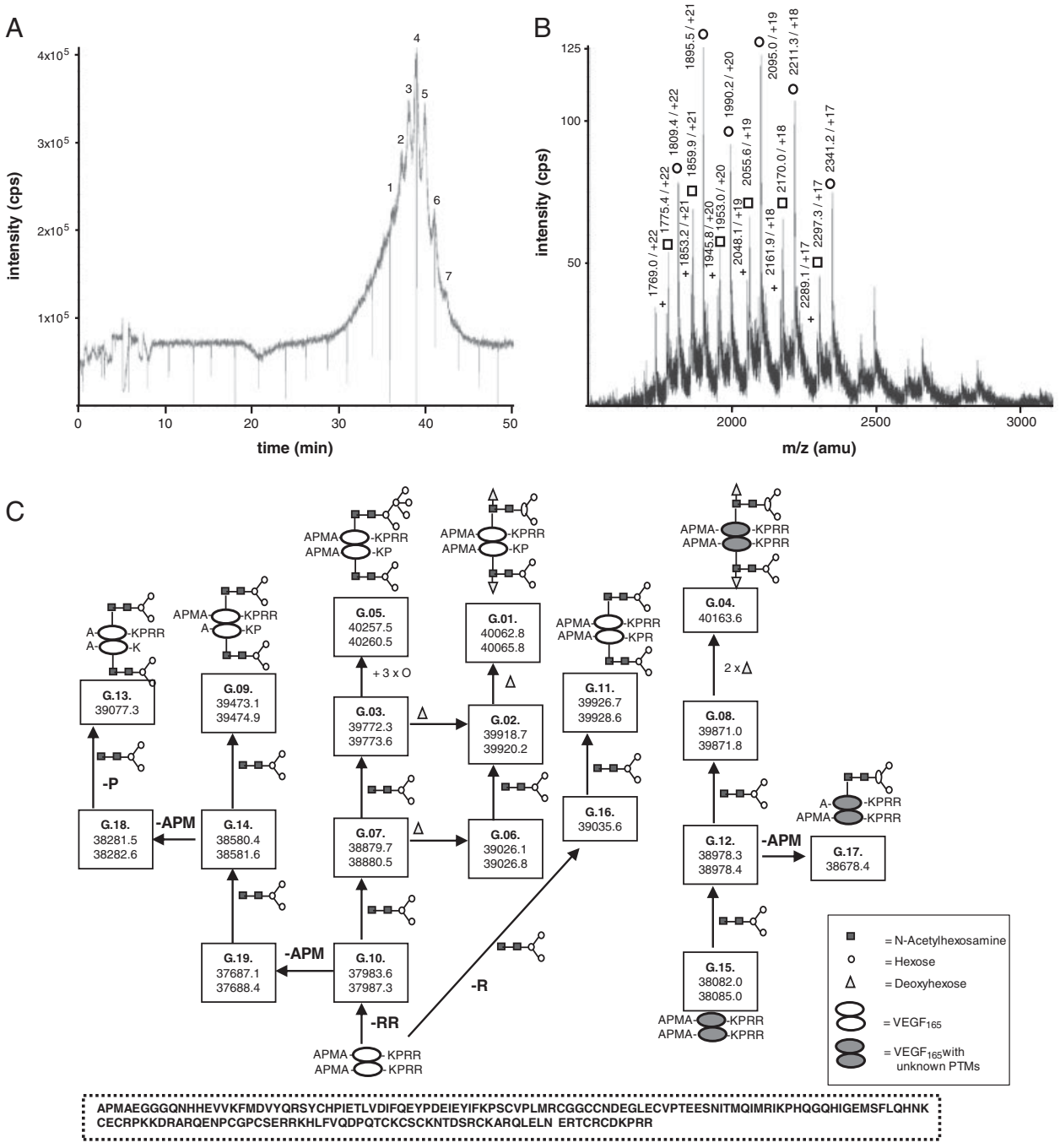


Figure 4. (A) CE-TOF-MS analysis of $VEGF_{165}$ on a polycationic polymer coating. (B) MS spectrum of time window 38.5–39.1 min in panel A (peak 4). Different *m*/*z* series are labeled in the figure (○,□,+). The *m*/*z* values and charge numbers are included. (C) Scheme of the different glycoforms 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]. Copyright Wiley-VCH Verlag GmbH & Co. Reproduced with permission.

2.2.2 Biopharmaceuticals

Other clinically relevant proteins, such as human growth hormone (hGH) and hemoglobin-based oxygen carriers (HBOCs), have also been examined by CZE-ESI-MS [7, 40–42]. hGH has been analyzed both on a negatively charged coating using a medium-pH 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

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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 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 ACN 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 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 CE (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 (Fig. 5A and B). The FACE-MS method was compared with 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–ACN–formic acid was applied and the non-covalent complex dissociated. Therefore, ammonium

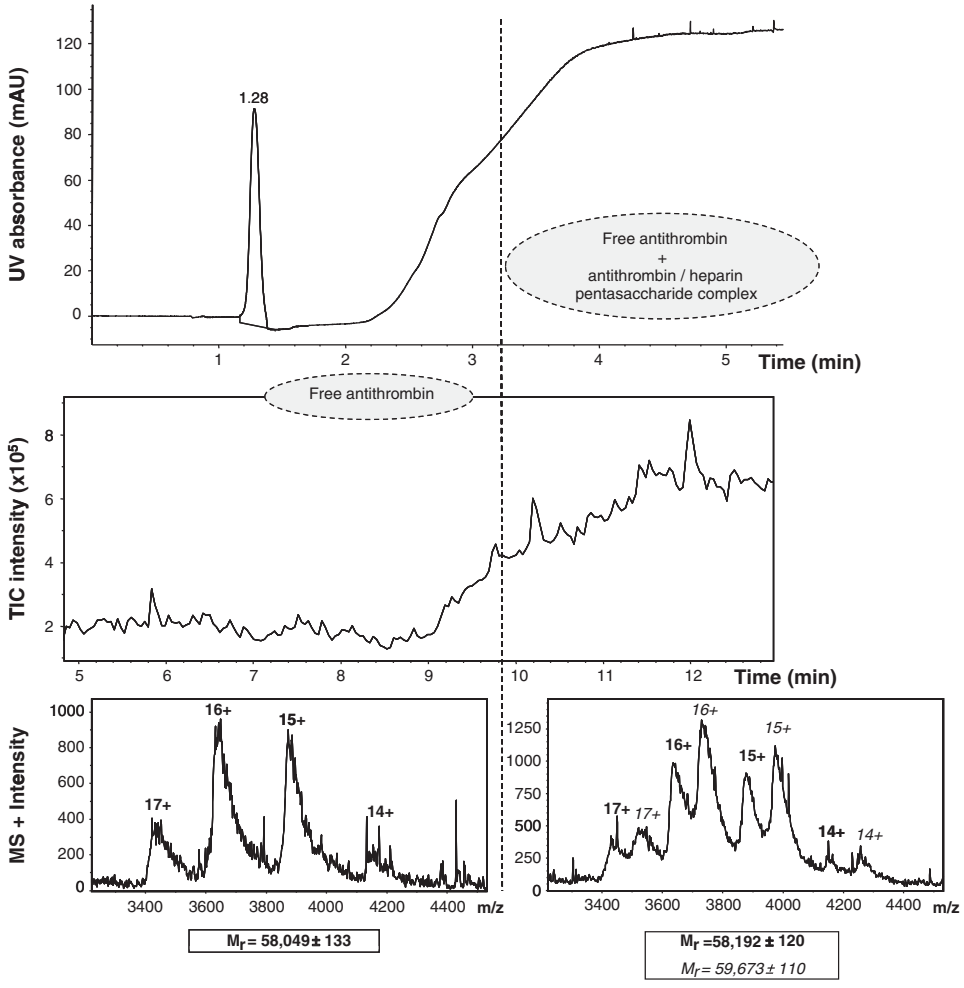


Figure 5. (A) UV absorbance electropherogram and (B) TIE of the FACE-ESI-MS analysis of the antithrombin–pentasaccharide interaction. Mass spectrum integrated over (C) 9–10 min and (D) 10–12 min time interval shows the presence of free antithrombin and the antithrombin–pentasaccharide complex. Antithrombin molecular mass is indicated in bold, the molecular mass of the antithrombin–-pentasaccharide complex in italic. Reprinted with permission from [8]. Copyright 2007 American Chemical Society.

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acetate (pH 6.5) was used as a non-denaturing sheath liquid. Under these conditions, good quality mass spectra were obtained that allowed identifying both the nonbound protein and the complex (Fig. 5C and D). A similar approach was used to get an insight into 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. Virtually for all studies the same CE method employing a low-pH formic acid BGE containing ACN 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 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 an average 1300 polypeptides with a molecular mass between 1 and 20 kDa (Fig. 6A). They also investigated the use of 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 (Fig. 6B and C), 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 ACN, isopropanol water and formic acid was applied to obtain efficient protein separations (Fig. 7A). 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 (Fig. 7B), which allowed the 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. In addition, 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 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 weights 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].

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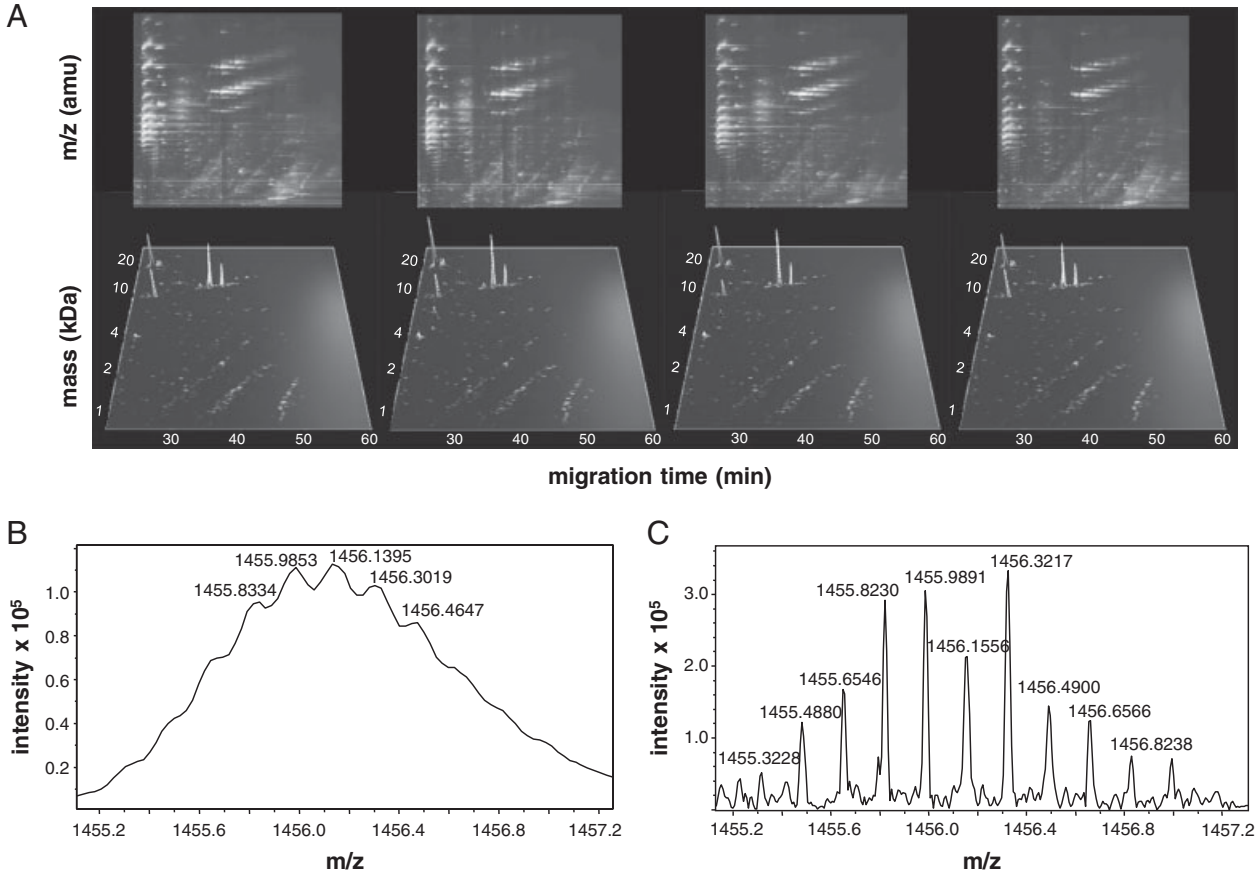


Figure 6. (A) Electropherograms obtained for 4 out of 19 consecutive CE-TOF-MS measurements of a single urine sample. The *m/z* values of the 2-D raw data plots (upper panel) and the molecular weight (logarithmic scale) of the deconvoluted 3-D plots (lower panel) on the *y*-axis are plotted against CE 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]. Copyright Wiley-VCH Verlag GmbH & Co. Reproduced with permission.

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). 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-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 40–60 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 homemade 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

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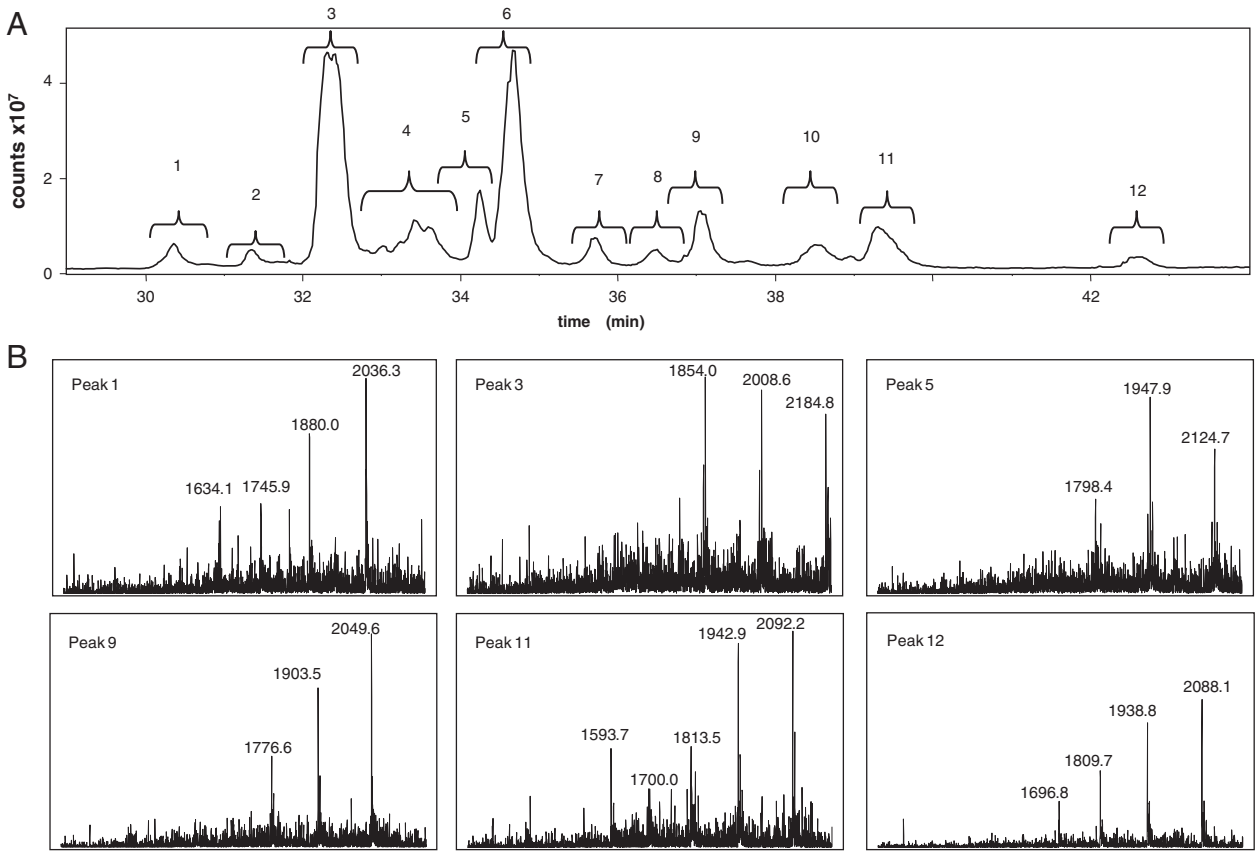


Figure 7. (A) Optimized CZE-ESI-MS separation of zein proteins using a BGE-containing ACN, isopropanol, water and formic acid (40/20/38/2, v/v/v/v). (B) MS spectra of six selected peaks depicted in panel A [59]. Copyright Wiley-VCH Verlag GmbH & Co. Reproduced with permission.

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 describe 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 analysis of the biopharmaceutical glucagon that was incubated with hydrochloric acid for 2 weeks (Fig. 8). Both glucagon and a single degradation product were baseline separated. The degradation product was identified as a single deamidation based on the observed p*I*-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 3). Some reports on the binding of metallo-drugs to 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] toward both proteins 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 a positively charged Polybrene (PB) coating in combination with a low-pH BGE [71]. They demonstrated that the binding of an Ru(III)-based drug with both proteins could be monitored under these conditions (Fig. 9A). The binding of the drug in patients (Fig. 9B and C) 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

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Table 2. 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]

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

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, α-lactoglobulin A; β-LGB, β-lactoglobulin B.

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

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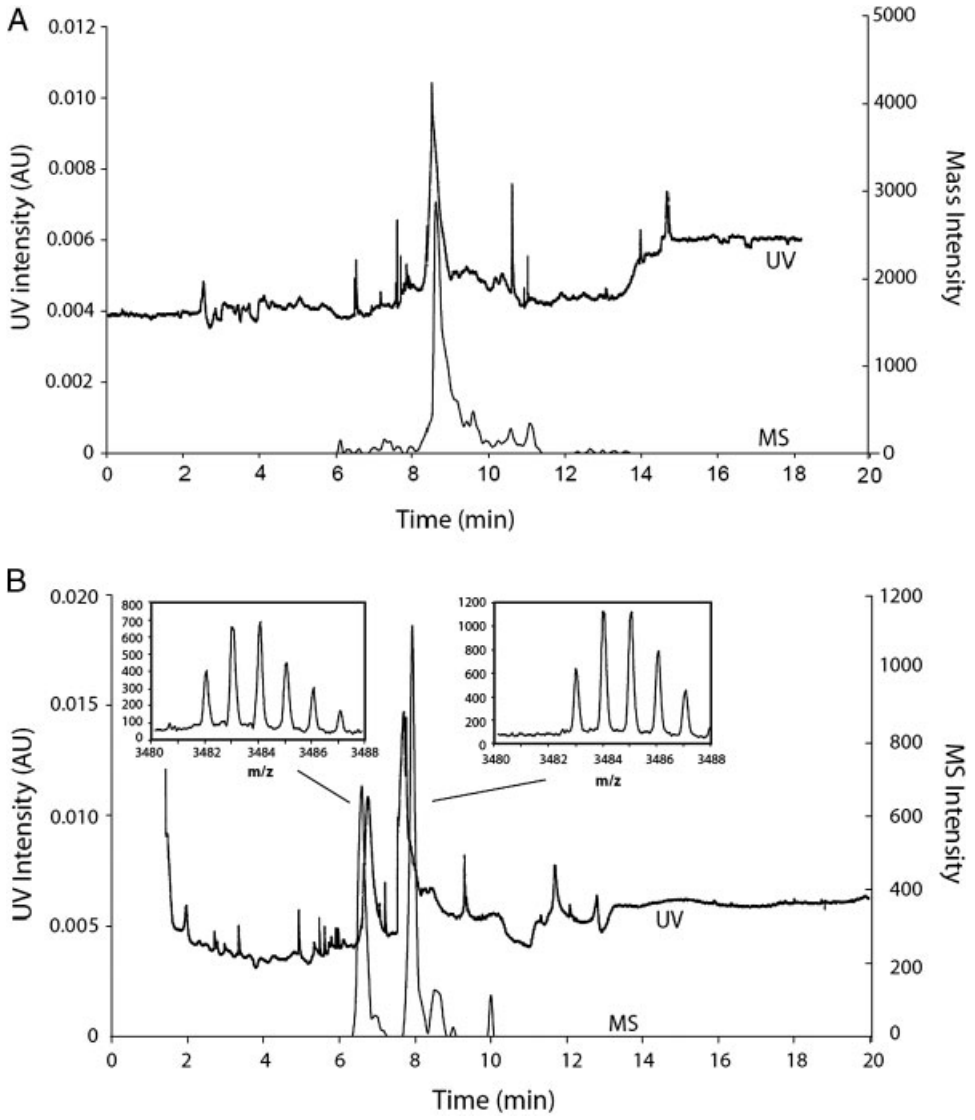


Figure 8. Reconstructed MS electropherograms (thin trace) and UV electropherograms (bold trace) of (A) intact glucagon and (B) degraded glucagon. Mass spectra of glucagon and the degradation product are shown in the insets [66]. Copyright Wiley-VCH Verlag GmbH & Co. Reproduced with permission.

Table 3. 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]

Note: 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.

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

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

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described by Bytzek *et 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 Concluding remarks

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 as 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 the detection of high molecular weight compounds, like mAbs [77]. Efficient CZE or CIEF separations may allow the 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.

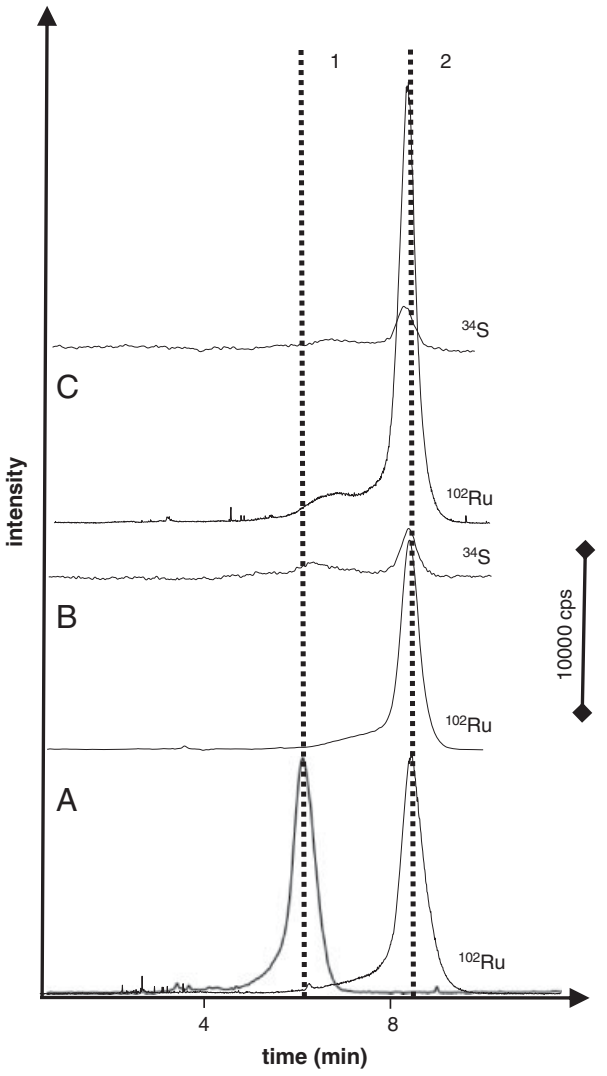


Figure 9. CZE-ICP-MS analysis of samples containing either Tf or HSA that were *in vitro* treated with an Ru-based anticancer drug (A). CE-ICP-MS analysis of a clinical sample from a patient on day 2 (B) and on day 18 (C) after first administration of an Ru-based anticancer drug. For each clinical sample, 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, whereas 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]. Copyright Wiley-VCH Verlag GmbH & Co. Reproduced with permission.

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.

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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 was 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 of 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.

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The authors have declared no conflict of interest.

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