Two-dimensional Capillary-based Electrophoretic Separation Coupled to Mass Spectrometry by a Mechanical Valve

Dissertation

der Mathematisch-Naturwissenschaftlichen Fakultät
der Eberhard Karls Universität Tübingen
zur Erlangung des Grades eines Doktors der
Naturwissenschaften
(Dr. rer. nat.)

vorgelegt von
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Tübingen
2017

The research described in this thesis was conducted between 01.09.2014 and 31.01.2017 at Aalen University, Faculty of Chemistry under the supervision of Prof. Dr. Michael Lämmerhofer and Prof. Dr. Christian Neusüß.

Gedruckt mit Genehmigung der Mathematisch-Naturwissenschaftlichen Fakultät der Eberhard Karls Universität Tübingen.

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“You sort of start thinking anything‘s possible if you‘ve get enough nerve.”

J.K. Rowling - Harry Potter and the Order of the Phoenix

Acknowledgment

An dieser Stelle möchte ich gerne denjenigen Personen danken, die mich auf dieser dreijährigen Reise durch all die Hochs und Tiefs begleitet und unterstützt haben. Besonderen Dank geht an:

Meine Betreuer; **Christian Neusüß** für die Möglichkeit der Promotion in deiner Arbeitsgruppe, die Unterstützung & Ideen und für die Teilnahme an mehreren internationalen Konferenzen. **Michael Lämmerhofer** für die hilfreichen und schnellen Antworten auf meine vielen Fragen und die große Unterstützung beim Verfassen des Review Artikels.

Julia Meixner dafür, dass du immer an meiner Seite warst, speziell in den anstrengenden Wochen der Messserien. Ohne dich würde ich heute nicht hier stehen.

Oliver Höcker, **Kevin Jooß**, **Cristina Montealegre**, **Sabine Neuberger** und **Jenifer Römer** für die Unterstützung in verschiedenen Projekten und die Hilfe bei der Reinigung und Reperatur unzähliger Geräte. Es war eine Freude, Teil dieses verückten Labors gewessen zu sein.

Kevin Jooß für das Korrekturlesen der Thesis, der vielen guten inhaltlichen Vorschläge und für die Anregenden (wissenschaftlichen) Diskussion.

Cathrin C. Heidsiek für tolle Gespräche, Tipps für San Francisco und das hilfreiche Feedbback zur Thesis.

Meinen Eltern dafür, dass sie immer zu mir gehalten haben, egal welche Entscheidung ich getroffen habe.

Andrea Beutner, **Jeannie Horak**, **Karin Krone**, **Jörg Schlotterbeck**, **Friedericke Sandbaumhütter** und **Sarah Wiesner** für die großartige Zeit im Rahmen der verschiedenen Konferenzen in Helsinki, Tübingen, Regensburg, Marburg, Hohenroda und San Francisco. Ohne euch wäre es nicht das gleiche gewessen und der Ausblick auf die nächste Konferenz motivierte mich im Laboralltag fokusiert zu bleiben.

Spotify für tausende von Stunden guter Musik.

List of Abbreviations

AC	Alternating current
BGE	Background electrolyte
BPE	Base peak electropherogram
CID	Collision-induced dissociation
CIEF	Capillary isoelectric focusing
CZE	Capillary zone electrophoresis
CE	Capillary electrophoresis
DC	Direct current
EACA	ϵ-aminocaproic acid
ESI	Electro spray ionization
EOF	Electroosmotic flow
FAc	Formic acid
GC	Gas chromatography
HAc	Acetic acid
Hb	Hemoglobin
Hb_{1C}	Glycated hemoglobin
HPLC	High-performance liquid chromatography
HPMC	(hydroxypropyl) methyl cellulose
IEF	Isoelectric focusing
IPG	Immobilized pH-gradient
kV	Kilovolt
LC	Liquid chromatography
LOD	Limit of detection
mAb	Monoclonal antibody
MALDI	Matrix-assisted laser desorption/ionization
MS	Mass spectrometry
m/z	Mass-to-charge ratio
nL	Nanoliter
PEEK	Polyether ether ketone
pH	Negative logarithm of hydrogen ion concentration
pI	Isoelectric point
PTFE	Polytetrafluoroethylene
Q	Quadrupol
SDS-PAGE	Sodium dodecyl sulfate - polyacrylamid gel electrophoresis
SEC	Size-exclusion chromatography
SL	Sheath liquid
TETA	Triethylenetetramine
TOF	Time of flight
UV	Ultraviolet

Abstract

Separation and identification are core disciplines in bioanalytics e.g. protein characterization. Capillary electrophoresis (CE) is a valuable technique for the analysis of intact proteins especially for the separation of their variants. Mass spectrometry (MS), providing high mass resolution and possible fragmentation experiments enables detailed identification and characterization of an analyte. However, low injection volumes and MS interfering components (ampholytes by applying capillary isoelectric focusing (CIEF) and involatile buffer utilizing capillary zone electrophoresis (CZE)) are in many cases drawbacks of CE separations. Tackling this drawbacks can be done in multidimensional setups. However, hyphenation of CE with CZE or HPLC is challenging due to high voltage, low transfer volumes and the need of dead volume free connections.

The general objective of this work was to enable an interference free MS detection for electromigrative separation systems, with focus on CIEF as a first dimension, which are interfering the electrospray ionization (ESI) process when hyphenated directly to ESI-MS. A thorough literature research on CIEF online hyphenated to MS and their applications was performed and revealed the demands, challenges but also the power of such a coupling. This literature research was the foundation for the development of the here presented multidimensional setups and placed the focus on the main objective of this work: the CIEF-CZE-MS hyphenation.

For the practical part, a 4-port-valve was utilized, which allows the coupling of CE and CZE-MS. Starting with model samples containing proteins and peptides, important parameters of the CE-CZE-MS setup were evaluated, e.g. a procedure to find the best transfer volume (between 4-20 nL) or the introduction of a multiple heartcut approach (up to 6 cuts in one run). Consequently, different applications like the separation and identification of charge variants of a monoclonal antibody by CIEF-CZE-MS and CZE-CZE-MS, utilizing the aforementioned 4-port-valve followed the establishment of the system. It was possible to characterize deamidation variants of a monoclonal antibody on intact level in a fast and straight forward way.

After the detailed evaluation of the aforementioned valve, it was also utilized for the promising nano HPLC(UV)-CZE-MS hyphenation for the separation of proteins and their corresponding variants. This combination of chromatographic and electromigrative separation mechanism was applied for the separation of a model protein mix in the first dimension. One of the obtained signals was detected as RNaseA/B in the LC(UV) dimension. This signal was further transferred into the CZE-MS dimension which enables the separation and MS detection of RNase A and RNase B including the different high-mannose variants of RNase B.

The main goal of this project was the development of different multidimensional setups including electromigrative separation for the application e.g. in the bio pharmaceutical field. This was successful especially in the case of the charge variant characterization of a monoclonal antibody on intact level. Moreover the intensive work on the coupling device allows further applications like with the mentioned nano HPLC-CZE-MS setup which will be pursued in following projects.

Zusammenfassung

Trennung und Identifizierung sind unverzichtbare Werkzeuge der Bioanalytik. Die Kapillarelektrophorese (CE) stellt dabei eine entscheidende Trenntechnik für Proteinvarianten auf intakter Ebene dar. Die Massenspektrometrie (MS) mit der Möglichkeit der Bestimmung der genauen Masse des Moleküls und weiterführenden Fragmentierungsexperimenten liefert hingegen wichtige Informationen zur Erkennung und Charakterisierung des Analytes. Kleine Injektionsvolumina und MS störende Bestandteile stellen entscheidende Nachteile von vielen CE Trennungen dar. Beispiele sind die MS störenden Ampholyte, die unumgänglich in der Kapillar-isoelektrischen Fokusierung (CIEF) sind oder spezielle Elektrolytsysteme in der Kapillarzonenelektrophorese (CZE), die nicht MS kompatibel sind. Multidimensionale Ansätze können diese Nachteile umgehen, allerdings ist die Einbindung einer CE Dimension in solch einen Ansatz aufgrund der hohen Spannung in der CE und der kleinen Übergabevolumina anspruchsvoll.
Ziel dieser Arbeit war die Entwicklung einer solchen multidimensionalen Plattform, um auch bei Trennsystemen, die MS störende Bestandteile enthalten, eine uneingeschränkte MS Detektion zu ermöglichen. Startpunkt des hier präsentierten Projektes war ein detailliertes Literaturstudium mit dem Fokus auf der online Kopplung von CIEF an MS. Diese Zusammenstellung und der Vergleich der recherchierten Literatur zeigte die Anforderungen sowie Limitationen bisheriger Ansätze, aber auch die Stärken einer solchen Kopplung auf. Darüber hinaus wurde diese Zusammenstellung als Grundlage für die Entwicklung der hier vorgestellten Plattformen genutzt und ergab gleichzeitig den Hauptfokus der Arbeit auf die Entwicklung einer CIEF-CZE-MS Kopplung.
Für den experimentellen Teil dieser Arbeit wurde ein 4-Wege-Ventil im Labor etabliert, welches die Kopplungen von CE an CZE-MS ermöglicht. Messungen zur Evaluierung des Systems wurden mithilfe eines Protein/Peptid Probenmixes durchgeführt. Parameter dieser Evaluierung waren unter anderem die Größe des Übergabevolumens sowie ein Multi "Heartcut" Ansatz zur Probenübergabe zwischen den Dimensionen. Anschließend wurden verschiedene Anwendungen wie die Charakterisierung von Ladungsvarianten eines Antikörper mittels CIEF-CZE-MS und CZE-CZE-MS durchgeführt. Deamidierungsvarianten eines Antikörpers auf intakter Ebene konnten detektiert werden.
Das oben erwähnte Ventil wurde nach der detaillierten Evaluierung auch für die Kopplung von nano HPLC(UV)-CZE-MS für die Trennung von Proteinen und deren Ladungsvarianten eingesetzt. Mithilfe dieser Kombination aus chromatographischen und elektromigrativen Trennmechanismen wurde in einem ersten Schritt in der LC(UV) Dimension ein Proteinmix aufgetrennt. Einer der detektierten Signale wurde dabei als eine Mischung aus RNaseA/B bestimmt. Dieses Signal wurde nachfolgend in die CZE-MS Dimension übergeben, in welcher RNase A von RNase B getrennt wurde. Darüber hinaus konnten auch verschiedene Mannose Varianten von RNase B charakterisiert werden.
Der Fokus des Projektes lag auf der Entwicklung multidimensionaller elektromigrativer Ansätze, welche in der Bioanalytik Anwendung finden sollten. Besonderes Augenmerk sollte dabei auf etablierte und bisher nicht MS zugängliche Trennsysteme gelegt werden. Dies konnte insbesondere für die Ladungsvariantentrennung eines Antikörper auf intakter Ebene erfolgreich durchgeführt werden. Darüber hinaus hilft die intensive Arbeit rund um das Kopplungsventil für die Etablierung weiterer Anwendungen, speziell unter Verwendung der nano HPLC-CZE-MS Plattform welche in zukünftigen Projekten eingesetzt werden soll.

List of Papers

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

I. **Capillary isoelectric focusing-mass spectrometry: Coupling strategies and applications.**
Jens Hühner, Michael Lämmerhofer, Christian Neusüß. *Electrophoresis*, 2015, 36, 2670-2686

II. **CIEF-CZE-MS applying a machanical valve**
Jens Hühner and Christian Neusüß. *Anal. Bioanal. Chem*, 2016, 408, 4055-4061

III. **Interference free mass spectrometric detection of capillary isoelectric focused proteins including variants of a monoclonal antibody**
Jens Hühner, Kevin Jooß and Christian Neusüß. *Electrophoresis*, 2016, doi: 10.1002/elps.201600457

IV. **Two-dimensional capillary zone electrophoresis mass spectrometry for the characterization of intact monoclonal antibody charge variants including deamidation products**
Kevin Jooß, **Jens Hühner**, Steffen Kiessig, Bernd Moritz and Christian Neusüß. *submitted to analytical chemistry*

Author's contribution to the papers:
Paper I - The general idea for the topic came from Christian Neusüß. I carried out the literature research, planned the structure, made the comparison of the different described techniques and wrote the main part of the article. Christian Neusüß and Michael Lämmerhofer were editing and proofreading the article.
Paper II - The idea for the CIEF-CZE-MS hyphenation came from Christian Neusüß. The development of the setup and the evaluation of the sample was performed by myself. Moreover, I planned and performed the experiments and wrote the article on my own. The proofreading and the final approval of the manuscript was done by Christian Neusüß.
Paper III - The experiments were planned by myself. I performed the main part of the work (90 %) including generation, analysis and interpretation of data with help from Kevin Jooß (10 %). The paper was written by myself with editing from Kevin Jooß. Christian Neusüß did the proofreading and act as corresponding author.
Paper IV - The idea for the work came from Christian Neusüß. Kevin Jooß, Christian Neusüß and I planned the work. The experiments were performed by Kevin Jooß (2/3) and myself (1/3). The paper was written by Kevin Jooß (2/3) and myself (1/3). Christian Neusüß was proofreading the article and did the final approval. Bernd Moritz and Steffen Kiessig were supporting the project by granting sample and proofreading the article.

Other papers not included in the thesis

- **Quantification of riboflavin, flavin mononucleotide, and flavin adenine dinucleotide in mammalian model cells by CE with LED-induced fluorescence detection.**
  Jens Hühner, Álvaro Ingles-Prieto, Christian Neusüß, Michael Lämmerhofer, Harald Janovjak. *Electrophoresis* 2015, 36, 518-525

- **Development and validation of a CE method for the characterization of sulfoethyl cellulose**
  Henrik Harnisch, **Jens Hühner**, Christian Neusüß, Andreas Koschella, Thomas Heinze, Gerhard K. E. Scriba *J. Sep. Sci.* 2016, 00, 1-8

List of oral presentations

I. **Quantification of FMN, FAD and Riboflavin in Different Cell Lines with CE-LED Induced Fluorescence Detection**
Jens Hühner, Michael Lämmerhofer, Christian Neusüß, Harald Janovjak
6^{th} CE-Forum, Marburg (Germany), September 29^{th} - September 30^{th}, 2014

II. **Fast and sensitive protein analysis with on-line coupled capillary isoelectric focusing - capillary zone electrophoresis - mass spectrometry**
Jens Hühner, Michael Lämmerhofer, Christian Neusüß
22^{th} International Symposium on Electro- and Liquid Phase Separation Techniques (ITP 2015) and 8^{th} Nordic Separation Science Symposium (NoSSS2015), Helsinki (Finnland), August 30^{th} - September 3^{th}, 2015
Metrohm best young science award for innovative research

III. **Fast and sensitive protein analysis with on-line coupled CIEF-CZE-MS**
Jens Hühner, Michael Lämmerhofer, Christian Neusüß
7^{th} CE-Forum, Tübingen (Germany), October 5^{th} - October 6^{th}, 2015

IV. **Capillary Isoelectric Focusing - Capillary Zone Electrophoresis - Mass Spectrometry Applying a Mechanical Valve**
Jens Hühner, Kevin Jooß, Michael Lämmerhofer, Christian Neusüß
42^{th} International Symposium on High Performance Liquid Phase Separation and Related Techniques (HPLC 2016), San Francisco (CA, USA), June 19^{th} - June 24^{th}, 2016

V. **Interference free mass spectrometric detection of capillary isoelectric focused proteins and variants of a monoclonal antibody**
Jens Hühner, Kevin Jooß Michael Lämmerhofer, Christian Neusüß
27^{th} phD meeting on separation science (Doktorandenseminar des AK Separation Science 2017), Hohenroda (Germany), January 8^{th} - January 10^{th}, 2017

List of poster presentations

I. **Quantification of FMN, FAD and Riboflavin in Different Cell Lines with CE-LED Induced Fluorescence Detection**
Jens Hühner, Michael Lämmerhofer, Christian Neusüß, Harald Janovjak.
30th International Symposium on Chromatography (ISC), Salzburg (Austria), September 14th - September 18th, 2014

II. **Fast and sensitive protein analysis with on-line coupled CIEF-CZE-MS**
Jens Hühner, Michael Lämmerhofer, Christian Neusüß.
7th CE-Forum, Tübingen (Germany), October 5th - October 6th, 2015

1 Introduction

Proteins and peptides are essential in all forms of life. Therefore understanding, identification, characterization and often quantification of these molecules is required in various disciplines. In this regard mass spectrometry (MS) represents a preferred analytical detection tool. Unfortunately, desired molecules are present in very complex biological samples containing over a thousand of different molecules. Therefore, an isolation of the molecule of interest from the rest is required prior to the detection. In case of large biomolecules electrically driven separation techniques are favored due to their matrix resistance, low sample volumes and high separation efficiency. The combination of electro driven separation and MS detection is a central topic of this work. For a better understanding of the here presented work, the introduction starts with a short explanation about proteins and peptides, followed by an introduction about the used separation and detection techniques. Subsequently the section about the hyphenation of separation techniques and detection leads to the results and discussion of the here presented work.

1 Peptides, proteins and monoclonal antibodies

Proteins represent the most versatile macromolecules in living organisms and are involved in nearly all biological processes. Proteins consist of amino acid chains, generated out of more than 20 different amino acids. The core structure of the amino acids can be found in figure 1.1 A. The residue "R" is individual for every amino acid. The linkage of the amino acids is implied in 1.1 B. The amino acid sequence is responsible for the 3D structure of the protein [1, p. 25]. Peptides (Figure 1.1 C1) are short proteins to a length of around 100 amino acids.
The analysis of structure and function of proteins and peptides is an issue since 200 years. In 1777 Pierre J. Macquer conclude all proteins as albumines. Despite primal separation achievements the structure of proteins was mainly unknown until the mid of the 20^{th} century. Better purification methods and a revolution in analytical technologies were leading to our contemporary understanding [2, p. 13].
Monoclonal antibodies (mAbs) (figure 1.1 C3) are complex high-molecular weight protein species which bind very specific on respective antigens. Two often identical light chains and two heavy chains, linked via disulfide bridges (dashed lines in figure 1.1) are the fundamental mAb structure. MAbs are more and more applied as pharmaceutical drugs, especially in cancer therapy and autoimmune disease treatment [3, pp. 201-205].

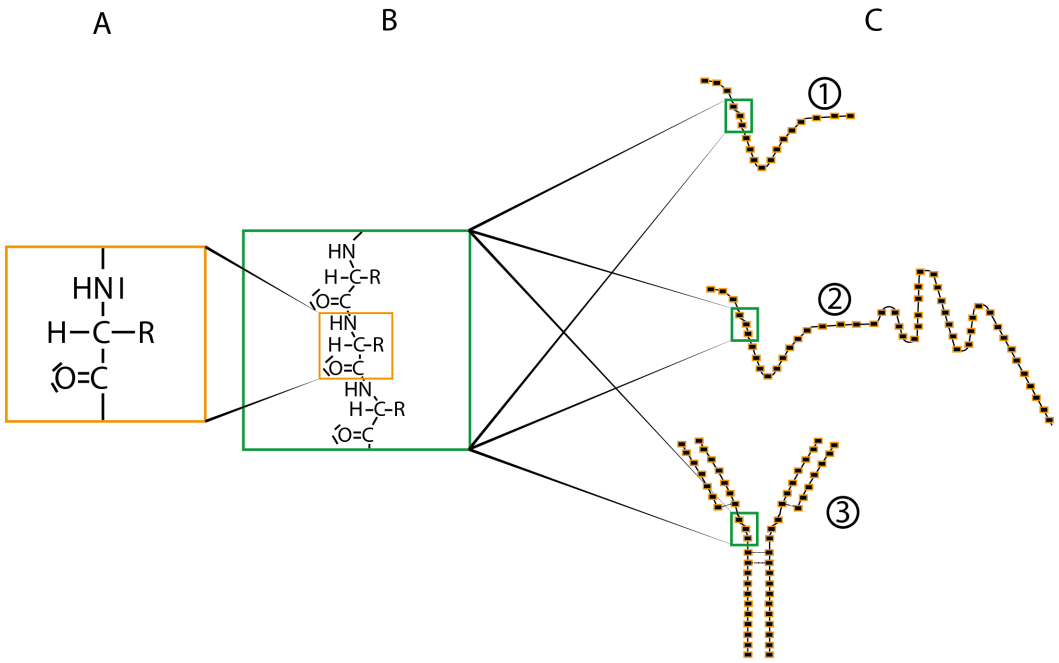

Figure 1.1: Structure of peptides (1), proteins (2) and mAbs (3). All three are built up by linkage (peptide bond) of amino acids (B) which have the core structure (A) with 22 different possible "R"s.

2 High performance liquid chromatography

In the last 25 years high performance liquid chromatography (HPLC) has become essential for the separation, purification and characterization of biomolecules. A basic HPLC system consists of a binary pump, micro degasser, sampling injection unit, column and a detection unit. In most applications a mobile phase is pumped with a constant flow (and a corresponding pressure) through the closed system. Liquid chromatography (LC) can be defined as the distribution of an analyte mixture between a stationary and a mobile phase. In general, an injected sample is pumped through the column to the detection unit. The time which the sample needed to the detection unit without any interactions with the stationary phase is called void time (t_0). Through interactions with the stationary phase the elution of the sample is delayed. This is called retention time (t_R). A universal and interdependent description of the retention of a component is given by the retention factor k [2]:

$$k = \frac{t_R - t_0}{t_0} \tag{1.1}$$

For the separation of two components, the respective k have to be different. Furthermore the selectivity α describes the ability of a system to separate two components.

$$\alpha = \frac{k_2}{k_1} \tag{1.2}$$

Nevertheless, to evaluate the quality of a separation, the peak width at baseline (w) have to be included. The quality is than given by R:

$$R = \frac{t_{R2} - t_{R1}}{\frac{1}{2}(w_1 + w_2)} \tag{1.3}$$

Thus, two peaks can be not ($R = 0$), partly ($R = 1$) or baseline ($R \geq 1.5$) separated. The band-broadening of a sample zone can be the result of different mass transfer phenomena and can be displayed by the reduced theoretical plate height (h) including the linear (u) or the reduced flow rate (ν):

$$h = A + \frac{B}{u} + C * u \quad or \quad h = A * \nu^{\frac{1}{3}} + \frac{B}{\nu} + C * \nu \tag{1.4}$$

This equation is called Van-Deemter- or Van-Deemter-Knox where A depends on the particle diameter and the packing factor, B on the diffusion in the axial direction and C on the mass transfer resistance. A good separation is characterized by a small reduced theoretical plate height (h).

Reversed phase LC

Reversed phase LC (RPLC) is the most commonly applied separation strategy for proteins and peptides. It uses the different hydrophobicity of the molecules for the separation. Thus, the sample molecules are loaded with a water based eluent onto the column, whereas the elution is caused by an organic solvent which is commonly pumped in a gradient mode. The stationary phase is based on porous or nonporous silica gel with nonpolar ligands (e.g. C_4, C_8 or C_{18} chains). The most accepted theory explaining the retention is the solvophobic model which states that the interactions are based on the exclusion of the mobile phase and the accumulation of the analyte at the stationary phase. Hence, the retention is based on the contact area of the molecule and stationary phase and the surface tension of the mobile phase [2].

3 Capillary electrophoresis

Applying capillary electrophoresis (CE), ions are separated according to their electrophoretic mobility by the application of an electric field. The electrophoretic mobility depends on the charge and the hydrodynamic radius of the ion as well as the viscosity of the background electrolyte [4]. It was first presented in fused silica capillary by Hjertén in 1967 [5] and applied to different samples by Jorgenson in 1981 [6]. An overwhelming success of CE was the sequencing of the human genome. Currently, it is also a vital analytical method in biopharmaceutical research with specific method for the majority of newly marketed biotechnology protein products [7]. Moreover, it was recently mentioned as the most important technology for the native mass spectrometric analysis of intact proteins [8].
The separation takes place in a fused silica capillary which is filled with a background electrolyte (BGE). Above a pH of 7 the silanol groups of the fused silica capillary are fully ionized. By applying voltage, the ionized H^+ ions and all other cationic species are migrating to the negative electrode and forming a flow which is termed electroosmotic flow (EOF). This EOF would also mobilize neutral molecules towards the negative electrode i.e. the cathode. In order to avoid the EOF and the adsorption of protein and peptides to the silica wall, the inner wall of the capillary can be coated with neutral polymers. The basic setup of a CE instrument is illustrated in Figure 1.2. In order to perform an analysis, the capillary is placed in the sample vial and part of the sample is injected by applying pressure or voltage. After injection the sample vial is replaced by the BGE vial and voltage is switched on. The injected ions are migrating due to their electrophoretic mobility. In the case of neutral inner coated capillaries only the positively charged molecules are migrating towards the outer vial and respective detector.

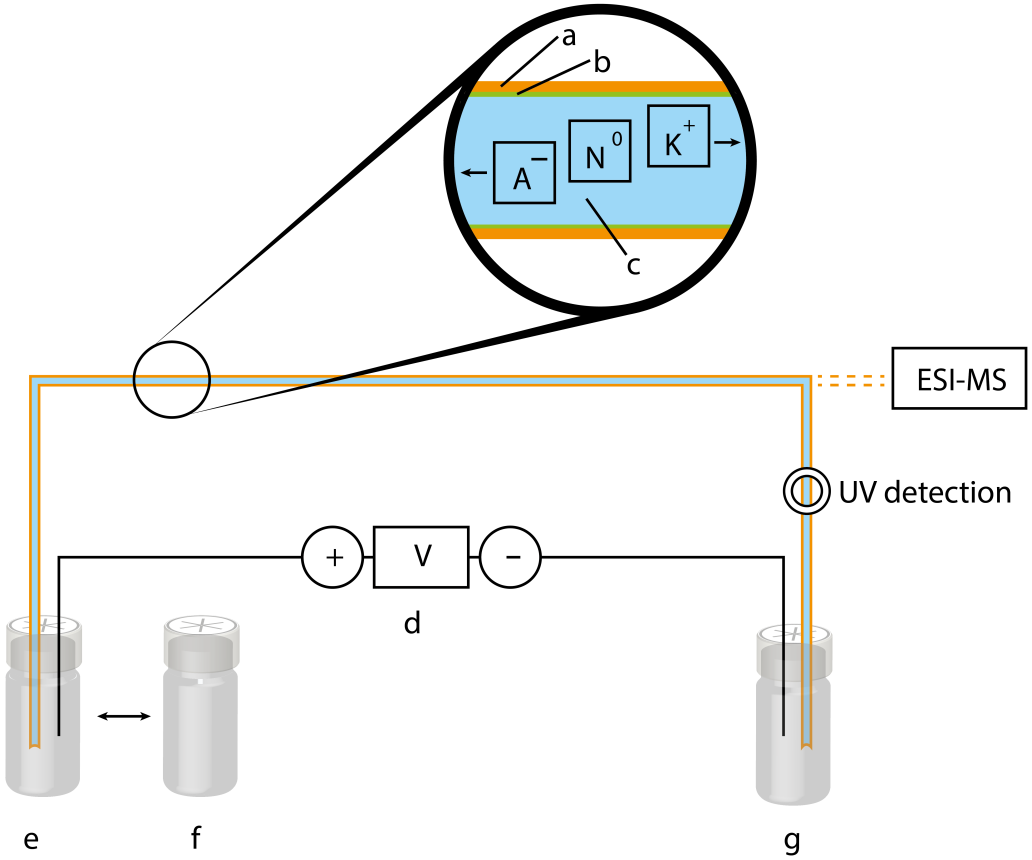

Figure 1.2: Scheme of a CE instrument including the fused silica capillary (a) with an polyimide outer coating and an neutral inner coating (b). The capillary is filled with a background electrolyte (BGE) (c) where the ions are separated. The capillary is placed in two vials containing the BGE (e and g). For injecting a sample, the inlet vial (e) is replaced by the sample vial (f). A high voltage source (d) is plugged to both vials to apply the separation or injection voltage. The outlet vial (g) is replaced through the electrospray ionization (ESI) interface, if MS detection is performed.

4 Capillary isoelectric focusing

Capillary isoelectric focusing (CIEF) works with the same instrumental setup as CE but a different separation mechanism is utilized (Figure 1.3). Isoelectric focusing (IEF) is applied for the separation of molecules according to their isoelectric point (pI). It was first shown in capillary by Hjertén and Zhu in 1985 [9]. Thereby, the capillary is filled with a sample solution containing ampholytes and spacers. By switching on the voltage, the ampholytes are generating a pH gradient. The spacers, which possess a pI of $<$ pH 3 and $>$ pH 10, respectively, are moving to both ends of the capillary and the sample molecules are focused where their own pI value equals the pH. This focusing step is illustrated in figure 1.3 b. After the focusing step is finished, the molecules are mobilized (see also figure 1.3 c) by inlet pressure or by exchanging the outlet vial to a vial containing an acid and applying voltage (c in figure 1.3).

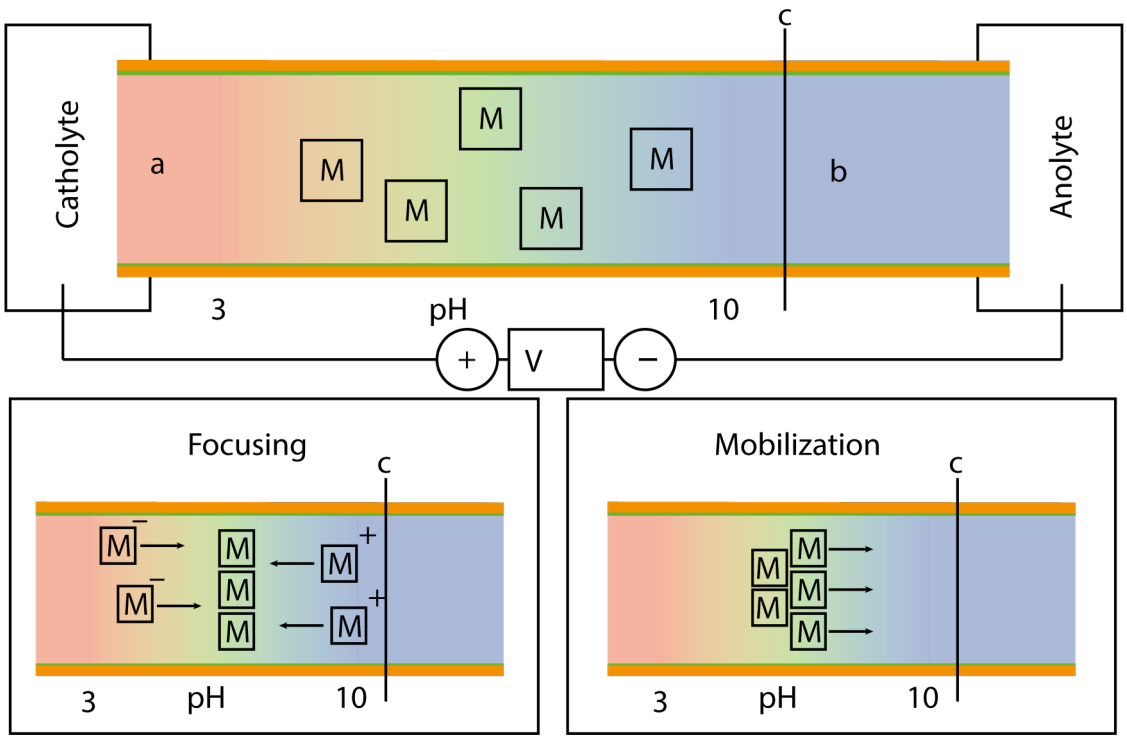

Figure 1.3: Scheme of the capillary isoelectric focusing process including the general setup (upper part), the focusing (lower left part) and the mobilization (lower right part).

5 Electrospray ionization process

In order to couple CE to mass spectrometry an ionization process is necessary due to the transfer of solutes into gaseous ions. The most commonly applied ionization technique for the hyphenation of CE to MS works with a three tubed coaxial sheath liquid sprayer (e) which is illustrated in figure 1.4. The capillary (c) is fixed in the center of the sprayer surrounded by a sheath liquid (SL) flow, which closes the electric circuit of the CE and the MS and assists the ionization process. In addition, a dry gas flow (a) supports the ionization process where the charged molecules are entering the process from the CE capillary forming very small droplets. These droplets loose neutral solvent over the time and the radius is reduced dramatically whilst the surface charges increase. Parts of the droplets explode (coulomb fission) and form smaller droplets while the process continuous. At the end gaseous ions are entering the MS. [4]

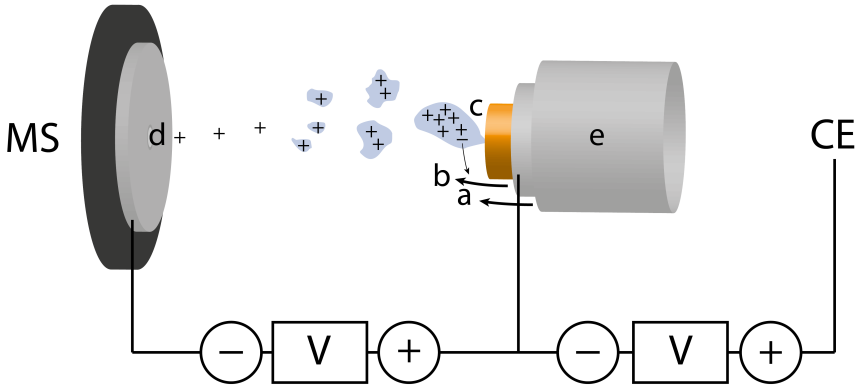

Figure 1.4: Three tubed sheath liquid sprayer (e) including dry gas flow (a), sheath liquid (b) and the CE capillary (c). The two necessary electric circuits of CE and MS are closed by the sheath liquid (b). The ionized molecules (here positive mode) are entering the MS through a hole in the end plate (d).

6 Mass spectrometry

Mass spectrometry instruments are utilized to separate ions according to their mass-to-charge ratio (m/z) with different mass analyzers. The principles of the here used MS instruments are explained in the next sections in detail. [2, pp. 380-382]

Time of flight mass spectrometer

A time of flight (TOF) mass spectrometer works by the electronic measurement of the time which ions need to traverse a defined distance in a high vacuum chamber. The ions get separated according to their (m/z) ratio because of the different speed initiated by an equal kinetic energy. The pulsation which is needed to determine a start point for the drift distance is achieved by a deflection due to an orthogonal aligned electric field. Typical drift distances are one to four meter. Advantages of a TOF-MS are the (theoretically) infinite mass range, the high mass resolution, and the fast acquisition rates. [2, pp. 382-385][10, pp. 132-136]

Quadrupole mass spectrometer

A quadrupole (Q) is a mass filter which allows only ions with a defined (m/z) ratio to pass the quadruple, which consist of four metal electrodes. The ions with the set (m/z) ratio are held by a combined AC/DC field on a stable path and pass the Q. Other ions are not able to pass the Q and collide with the electrodes. The Q-MS is a cheap and robust mass analyzer and ideal for ion selection. [2, pp. 385-388]

QqTOF mass spectrometer

A QqTOF instrument combines the advantage of both mass analyzer and is displayed in figure 1.5. The first Q (c) works as a mass filter, while the second q (d) is used as a collision-induced dissociation (CID) cell. Prior the first Q the ions are focused and transferred from the ESI process through a transfer capillary and an ion funnel towards the first Q. The acceleration (e) the flight tube (f), the reflectron (g) and the digitizer (h) represent the TOF stage providing high mass resolution and accuracy in short analysis time combined with a broad mass range. [2, pp. 395-397]

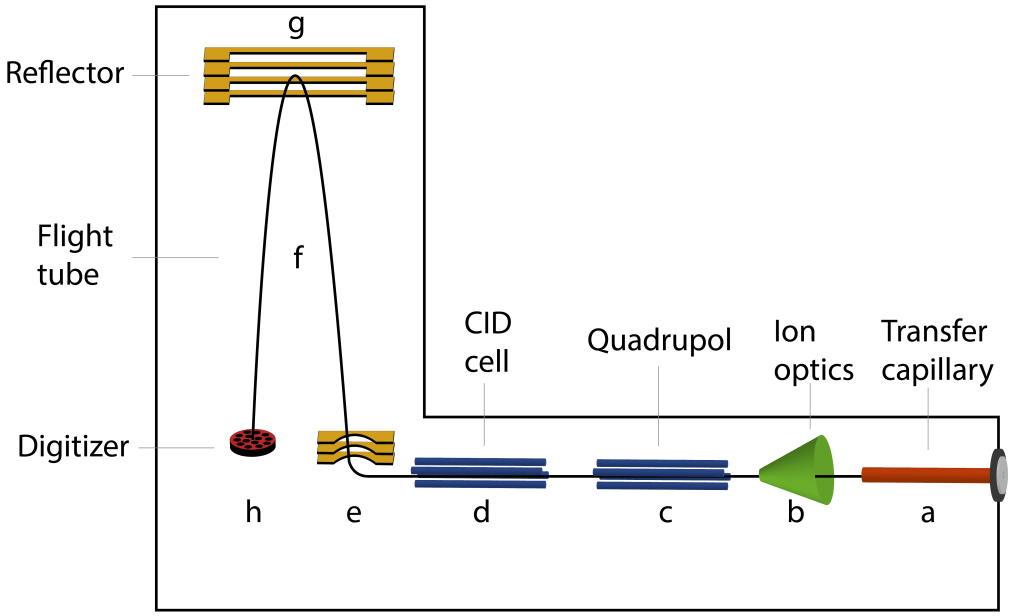

Figure 1.5: Sketch of a quadruple quadruple time of flight mass spectrometer. Molecules which are entering after the ionization process (not shown) were transported through the transfer capillary (a) and both quadruples (c+d) with a focusing step in the ion funnel (b). After the acceleration (e) into the flight tube (f) the molecules were detected on the digitizer (h).

7 Hyphenation of two or more separation techniques

Multidimensional separation is a commonly applied strategy for the analysis of complex samples due to the combination of different selectivities [11]. This combination can be used to improve the resolving power or to separate between an analyte and a component from a first dimension prior to a further dimension. Different kinds of transfer strategies can be utilized to transfer one or more desired analytes from one to another dimension: i) Single heart-cut, where only a single fraction of one dimension is transferred, ii) multiple heartcut, where more than one fraction is transferred, and iii) comprehensive (indicated by the letter '"X'"), where the entire first dimension is transferred into the next dimension [12]. There are a lot of existing and well established coupling techniques such as LCxLC or two dimensional gas chromatography (GCxGC). Using an electrophoretic separation in such a multidimensional technique is obvious due to high separation efficiency, the broad range of separation mechanism, the low liquid consumption and the low cost per analysis [11]. Nevertheless, a coupling of two electrophoretic separation techniques is challenging due to small transfer volumes and the required close to zero dead volume.

8 Intact protein separation for top-down proteomics

The field of proteomics, the analysis and characterization of the proteome of an organism, was announced as the next important challenge after the successful completion of the human genome project [13]. Berry L. Karger, an expert for the last 50 years on separation science, recently declared that the analysis of the proteome is the challenge of the 2010s

years and will be followed by the analysis of variants of the proteins in the 2020s [8]. For both, protein and related variant analysis, separation techniques have to be coupled to high resolution MS detection for an unambiguous and fast identification. In the field of protein variant characterization the analysis on intact level, termed "top-down analysis" is of major interest. Top-down is defined by the preservation of intact structure of proteins during separation. The opposite approach, referred to as "bottom-up" analysis, starts with the digestion of the protein by enzymes to peptides prior to the MS analysis. This approach allows e.g. the coverage of the protein sequence by database comparison and is not in the scope of this work [14]. In Figure 1.6 the most important techniques applied in the analysis of intact proteins and the field of application are summarized. Size exclusion chromatography (SEC) and 2D gels are considered as the gold standard technique while RPLC and capillary zone electrophoresis (CZE) are the most promising techniques as especially due to the hyphenation possibility to high resolution MS [13].

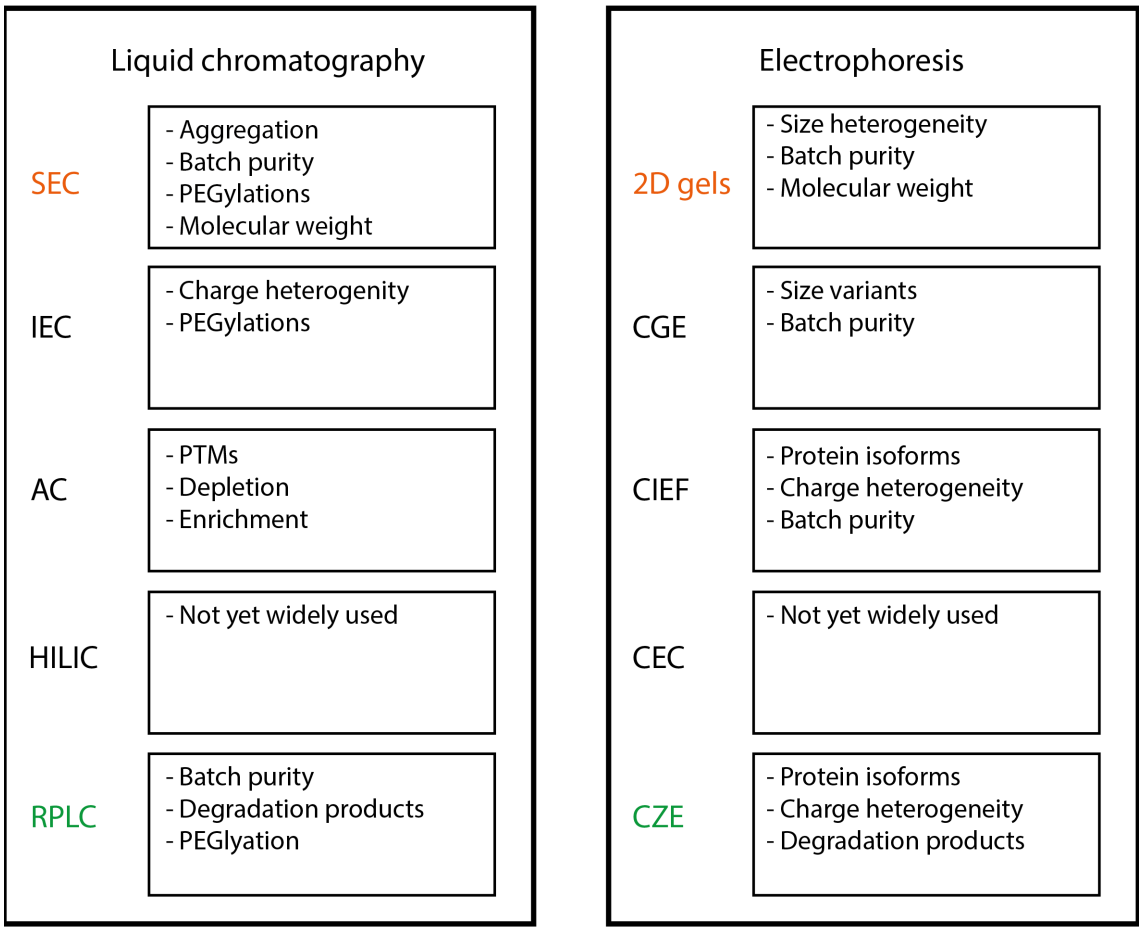

Figure 1.6: Liquid chromatographic and electrophoretic methods applied for top-down proteomics research. Indicated in orange are the techniques considered as gold standard for intact protein analysis. Highlighted in green, the most promising technique [13][15] (IEC = ion exchange chromatography; AC = affinity chromatography; HILIC = hydrophilic interaction chromatography; CGE = capillary gel electrophoresis; CEC = capillary electrochromatography).

A major field for protein and protein variants characterization represents the pharmaceutical and biomedical analyses. In 2013, protein biopharmaceuticals represented 20 %

of the total pharmaceutical market [15]. Regarding biopharmaceuticals, it is of utmost importance to characterize the different occurring protein variants, due to their potential influence on the biological and clinical efficacy [13].

9 Hyphenation of CIEF and MS

If the pI value and the mass of a molecule is required, isoelectric focusing (IEF) in immobilized pH gradient (IPG) strips is often combined with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) [16]. This 2D separation was subsequently expanded by offline structural characterization and identification via MS and data base search [17]. Extensive method run times and manual handling steps lead to its replacement by other methods (e.g. nanoLC-ESI-MS/MS of tryptic digests of the whole proteome-shotgun proteomics) [18]. The replacement of nano-LC by CIEF online hyphenated to MS is hindered by practical complications such as ESI interfering substances required for the CIEF process. The majority of ESI interfering substances is covered by the ampholytes, which are necessary to generate the pH gradient of the CIEF separation [19]. Furthermore the applied strong acids and bases are often non-volatile and causes instabilities in the electrospray process [20]. If direct coupling between CIEF and MS is performed, the operator is confronted with issues related to signal suppression and ionization efficiency. Therefore, a compromise between separation efficiency and signal intensity in the MS detection has to be accepted if applying a direct coupling of CIEF and MS. In order to avoid this compromise, different approaches such as matrix-assisted laser desorption/ionization (MALDI) as an alternative ionization technique, an immobilized pH gradient or the separation of ampholytes and analytes prior to MS have been pursued and will be discussed in more detail in the following subsections:

CIEF coupled to MALDI-MS

A crucial step connecting CIEF separation to MALDI-MS represents the transfer of the focused analytes onto the MALDI plate without losing the separation efficiency. The small peak volume of focused sample zones demand an addition of sheath liquid to achieve efficient sample droplet deposition [21]. However, MALDI is less sensitive for additives such as salt or ampholytes (signal decrease of 50 % by applying 1 % ampholyte) [22]. The first offline CIEF-MALDI-MS interface was reported in 1995 [23]. Foret and coworkers were applying a tee union, which is shown in Figure 1.7 A1, to admix the sample with the sheath liquid and to connect the electrode at the cathodic side. The CIEF capillary was placed inline in front of 60 collecting capillaries. The detection via ultra violet (UV) light was carried out shortly prior to the tee connection. The analytes were stored as solutions or salt free solids. 2 μl of the stored sample was deposited onto the matrix prespoted MALDI plate. This setup was improved by the same group by applying two UV detectors close to the sheath flow interface. Lechner et al., were utilizing a system which enables a direct deposition of the droplets onto the MALDI plate [21]. The sheath flow interface was located inside the cathodic vial during focusing and was placed briefly over the MALDI plate during mobilization. The mobilization/deposition step is illustrated in Figure 1.7 A2. Another possibility to spot analytical bands on a MALDI plate is to separate the electrode via a membrane shortly before the outlet of the capillary [24]. This interface can be found in Figure 1.7 A3. The MALDI plate was precoated by parafilm M and prespotted by MALDI matrix. In conclusion, the offline approaches for the coupling of CIEF to MALDI-MS enables further analysis including Edman sequencing, however, these systems suffer from long analysis times and tedious lab work. The development of an online spotting was a great effort and was extended by the sheathless spotting from Zhang

et al. which results in a higher concentrated sample on the MALDI plate. Nevertheless all aforementioned concepts are associated with limited resolution and sensitivity for larger analytes, interference from ampholytes and limited separation efficiency under more MS friendly conditions.

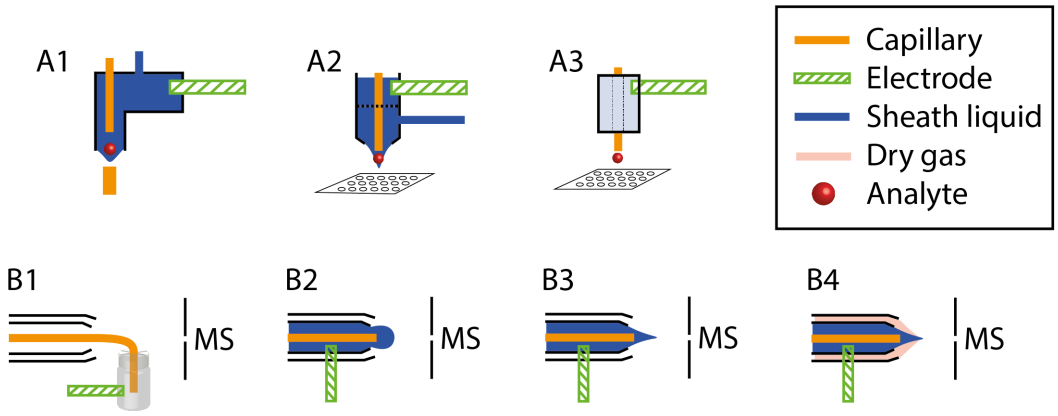

Figure 1.7: From A1 to A3: different CIEF-MALDI interfaces. A1: storage in capillary and offline transfer to MALDI plate. A2 and A3: Online deposit of sample to MALDI plate with (A2) and without (A3) sheath liquid. B1 to B4: direct CIEF-MS coupling with catholyte/spray solution exchange by placing a catholyte vial in the ESI housing (B1), using the sheath liquid as catholyte solution during the focusing (B2) and as sheath liquid during the ionization (B3) or by applying the same solution as catholyte during the focusing and as sheath liquid to support the ionization (B4).

CIEF-MS with catholyte/spray solution exchange after focusing for mobilization of analytes into MS

The first direct coupling of CIEF to ESI-MS by placing the catholyte vial inside the electrospray housing was demonstrated by Tang et al. [19]. This setup, including the catholyte vial and the electrode, is schematically outlined in Figure 1.7 B1. The mobilized analytes were monitored by UV detection. With this setup the influence of the ampholyte concentration was determined. A reduction of the ampholyte concentration from 5 to 0.5 % results in signal increase of 22 %. They demonstrate that the principle of direct CIEF-ESI-MS works, however, this approach is not automated and the influence of ampholytes is still a major concern. To avoid the placement of the cathodic vial inside the electrospray housing (B1), Clarke et al. were delivering the cathodic solution via the sheath liquid channel of a coaxial sheath liquid sprayer (B2) [25]. In this way, the cathodic solution is generating a droplet at the tip of the capillary. For mobilization and MS detection the cathodic solution is replaced by the sheath liquid which assisted the ESI process (B4). The direct CIEF-MS setup invented by Tang et al. was applied for different biological samples receiving impressive results, e.g. the studies from Smith and Jensen et al. [26, 27, 28]. They detected 210 discrete peaks or 900 unique putative protein masses. However, the ionization interfering effect of the ampholytes still remains. Moreover, all applications utilizing this setup required one replacement step and a voltage interruption, which potentially result in a loss of separation efficiency and complicating automation.

Direct CIEF-MS

In order to avoid the replacing step mentioned above, Zhang et al. utilized the same solution as catholyte during the focusing (Figure 1.7 B3) and as sheath liquid during the mobilization, ionization and MS detection (B4) [29]. In this way, they were providing a fully automated process for the analysis of focused analytes. Nevertheless, a separation between analytes and ampholytes was not achieved and therefore, interference during the ESI process could not be prevented.

CIEF-MS with partial filling, multiple junction capillary and integrated mircodialysis unit

A partial filling of the CIEF capillary results in discontinuous ampholyte zones. This is shown in Figure 1.8 A were the two ampholyte zones (A) are positioned in front and behind the sample (S in Figure 1.8 A) zone. This allows a mobilization into the MS where the sample zone enters the ion source without ampholytes [30]. Another possibility to avoid the transport of ampholytes into the MS is provided by a multiple junction capillary, where the capillary is separated into sections with a well defined pI cut off value joint via membrane insertion (B in Figure 1.8) [31]. This setup allows local buffer exchange and voltage gradients. A related technique represents the application of a dialysis interface prior to MS (C) [32]. With a membrane cut-off of 650 Da, it is possible to separate between the small ampholyte molecules and the macromolecular analytes. In conclusion, all these concepts showed an interesting idea to separate between ampholytes and analytes, however, they were not applied for a wide range of or more complex analytes, up to now.

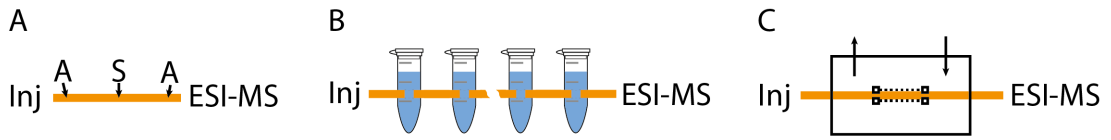

Figure 1.8: Different CIEF-MS interfaces, which separate between sample and ampholyte prior to MS. A: By the partial filling of ampholytes (A) before and after sample (S). B: by isoelectric focusing in a multiple junction capillary. C: supported by a dialysis interface.

CIEF-LC-MS

A successful way to get rid of all interfering substances from CIEF prior to MS detection is to implement an LC separation between CIEF and MS. In order to online couple CIEF to an LC-MS system, a valve, a membrane, or a dialysis interface is necessary to connect the electrode to the CIEF system. Chen et al. were utilizing a 6 port valve to achieve a connection to the LC-MS instrument on the one hand, and, to close the electric circuit from CIEF on the other hand [33]. This setup is illustrated in Figure 1.9 A. The analytes were transferred to the LC by pressure mobilization and a 400 nL sample loop, connected to the 6-port-valve. A trap column was used to separate between the most of the ampholytes and the analytes. Another possibility to close the electric circuit while connecting the LC system is to integrate a tee-union and a membrane (see Figure 1.9 C) [34]. This design was extended by the inventors years later by the use of 10 sample storage loops (C) [35], which improves the sampling process. Later, the tee piece was replaced by a dialysis membrane [36]. Moreover, Wang et al. additionally included a micro reactor (MR in Figure 1.9 d) for online digestion prior to the LC-MS analysis. Summarized, the presented CIEF-LC concepts were allowing to fully separate between ampholytes and analytes and were

applicable to complex samples such as cell lysates of *Chlorobium tepdium*. Nevertheless, all concepts are associated with long run times and complex instrument set up, including valves, pumps, columns, storage loops and dialysis membranes.

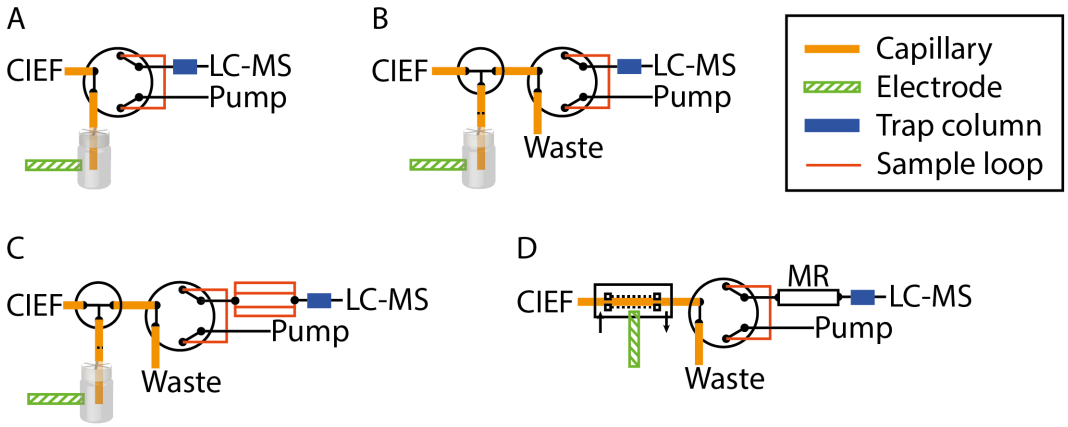

Figure 1.9: Different CIEF-LC-MS setups including one six-port-valve (A), the combination of six-port-valve and tee-union (B), the addition of storage capillaries (C) and membrane interface (D).

Other CIEF-MS interfaces

Another possibility to avoid the introduction of ampholytes into the ESI-MS is to immobilize them. In this particular case the ampholytes were immobilized on a monolith inside the capillary [32]. The outlet end of the capillary was mounted inside a coaxial sheath liquid sprayer, where the sheath liquid acts as catholyte solution and sheath liquid to support the ionization process. This approach shows an alternative for the separation between ampholyte and analytes, however, it is also associated with some drawbacks including significant loss in efficiency due to dispersion effects of the focused bands with the monolithic bed. Alternatively, separation between ampholyte and analyte was also accomplished by the application of free flow electrophoresis [37] or asymmetric flow field flow fraction [38]. Up to date, both concepts have only been applied for model analytes, suffer from complex instrument set up and make it in general impossible to calculate the transferred sample volume.

By concluding the previous CIEF to MS hyphenations, direct CIEF-MS coupling and CIEF-LC-MS coming up with the highest number of applications. The direct coupling shows a greater dynamic range and a higher throughput in comparison with 2D flat gel IEF coupled to gel sieving electrophoresis approaches. It also allows the work with intact proteins in a close to native environment. This is especially interesting for top-down analysis. However, due to the ampholyte concentration a compromise has to be made between separation efficiency in the CIEF process and signal intensity in the MS detection. The CIEF-LC-MS coupling was also applied with great success but nevertheless is associated with complex set up and long run times. Thus the search for a simple and reliable CIEF-MS system which prevents the high resolving power from the focusing process and offers highly sensitive MS detection continues. This goal is of great interest in context of increasing performance and availability of high-resolution MS options for intact protein analysis. It is also a great instrument combination for the characterization of mAbs, which are currently the most raising molecule class used as pharmaceutical drugs.

10 Hyphenation of LC and CZE-MS

As stated before one of the great analytical challenges in the next years is the sensitive characterization of protein variants. Relatively (to CE) large injection volumes and robust separation efficiency for proteins provided by LC in combination with the high separation efficiency for protein charge variants on intact level given by CZE could be a promising answer to this challenge. Especially due to the development of robust and commercially available nano LC systems, the volume difference between LC and CZE is in a reasonable range.

2 Objective of this work

Many electromigrative separation methods which are frequently used in the bioanalytical field (e.g. for the characterization of mAbs on intact level) are only available with UV detection due to ESI-interfering substances. The main objective of this project was to achieve interference free MS detection through multidimensional setups for such electromigrative separation methodologies with focus on CIEF separation as a first dimension.

The general work can be divided into three parts:

i An intensive literature search on multidimensional electromigrative setups with focus on online CIEF-MS hyphenations.

ii The development of a mechanical coupling device for CE-CZE-MS with focus on CIEF-CZE-MS, the validation with model samples followed by applications to demonstrate the power of such systems.

iii The utilization of the above used coupling device for the combination of chromatographic and electromigrative separation in a nano LC-CZE-MS setup.

At the beginning the intensive literature search was carried out to specify the demands and on the other hand the challenges of a 2D electromigrative setup with focus on CIEF utilized in the first dimension. In parallel the collected information was used to compile a review article (**Paper I**) concerning this topic.

The main part of the project was the implementation of the CIEF-CZE-MS and CZE-CZE-MS systems including the validation by applying protein and peptide mix samples. The characterization of charge variants of a monoclonal antibody as an application was utilized to demonstrate the power of the setup. The results are described in **Paper II, III** and **IV**.

Finally the previously utilized and validated coupling device was applied for a nano LC(UV)-CZE-MS setup for the characterization of proteins and their corresponding variants. This platform combines the advantage of column chromatography (large injection volume; preconcentration) and CE (suitable for charge variants) with MS detection which leads to a separation according to hydrophobicity (LC), charge & hydrodynamic radius (CZE) and mass to charge ratio (MS) in one analytical run.

3 Results and Discussion

The results and discussion chapter commences with an introduction of our own CIEF-CZE-MS setup applying a mechanical valve. This CIEF-CZE-MS setup was evaluated by a proof-of-concept study leading to the first application (MS characterization of CIEF separated hemoglobin and the glycated form) and is discussed in section 2 and 3. Consequently, the development by e.g. the introduction of multiple heart-cut transfer of analytes was leading to the MS determination of focused variants of a monoclonal antibody which is discussed next. Furthermore, the same antibody was alternatively characterized by a CZE-CZE-MS system utilizing the same mechanical valve (section 7). In addition, this 4-port-valve was successfully applied for the promising hyphenation of nano LC and CZE-MS for the separation of proteins and their corresponding variants. In the final chapter the development of an alternative hyphenation device for electrically driven or microfluidic separation techniques is described, concerning the limitations of the previously utilized 4-port-valve.

1 CIEF-CZE-MS: separation between ampholytes and proteins or peptides

A main goal of the here presented work was the development of an interference free MS detection of CIEF separated analytes. For the implementation of such a CIEF to MS coupling concept the most upcoming challenge, the separation of the analyte from the ESI interfering ampholytes has to be solved first. As previously mentioned, this is in principle possible utilizing an LC system or a dialysis interface in front of the MS detection. Nevertheless, dilution, adsorption effects and long run time are major drawbacks of both concepts. On the other hand, CZE is considered as an appropiate separation technique placed between CIEF and MS, due to the same capillary dimension as CIEF and the well established coupling to MS via a coaxial sheath liquid interface. A separation between ampholytes (in this case Servalyt^{TM}) and protein (in this case hemoglobin) utilizing CZE is possible applying a 0.2 M formic acid (FAc) BGE. A base peak electropherogram (BPE) of such a separation is shown in Figure 3.1.

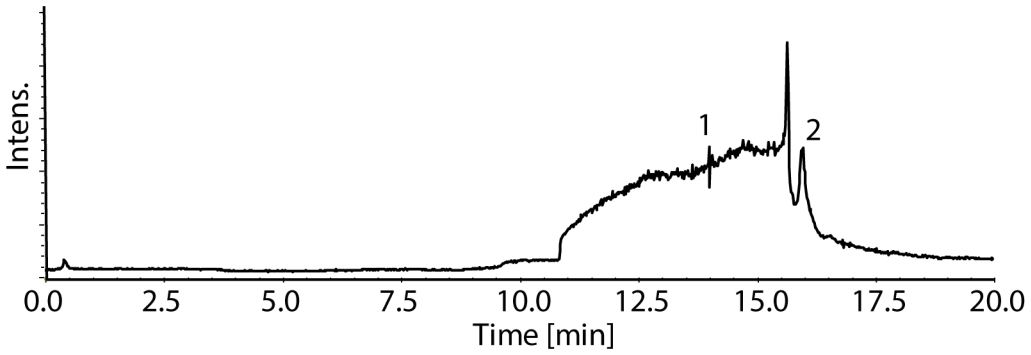

Figure 3.1: Separation between ampholytes (1) and protein (2) applying CZE. BGE: 0.2 M FAc; 10 kV for 20 min; 40 cm neutral coated capillary (ID 50 µm). Injection (20 nL) of hemoglobin in 3-10 ampholyte solution (Servalyte^{TM} 3-10; 1 % v/v). The MS parameter were set to achieve sufficient sensitivity for both, proteins and ampholytes.

2 4-port-nL-valve as interface for 2D electromigrative driven setups

After the general capability to separate ampholytes and protein via CZE was ensured, a concept such as CIEF-CZE-MS was aimed to enable MS detection of CIEF separated proteins without the interference of ampholytes. However, this setup requires the coupling of CIEF and CZE, which was, to the best of my knowledge, not achieved so far. An interface for the hyphenation of two electromigrative separation devices requires to fulfill certain demands: It has to be electrically isolated, requires low dead volume connections and a small transfer volume. All three requirements are fulfilled by the 4-port-nL-valve which is displayed in Figure 3.2. The valve consists of three parts: a stator, a rotor and a motor. The stator allows the connection of the capillaries (C) to the valve with finger tight screws (B). The rotor provides two small and one larger loop. The larger loop, further refereed as sample loop, has an internal volume of 4 - 20 nL, depending on the applied rotor, and is utilized for transferring a specific analyte from the first into the second dimension. The motor enables the switching between the two possible valve positions. The electrically isolated stator and rotor is made out of polyether ether ketone/polytetrafluoroethylene (PEEK/PTFE) material. This polymer mixture provides on one hand the needed chemical resistance and on the other hand a sufficient dielectric strength (22 kV/mm). The mentioned 4-port-valve was already in the lab [39], however, no reproducible work was possible due to current breakdowns and leaking problem. Several development steps, e.g. current limitation due to the material and focus on the capillary connections which were performed by myself, were leading to its utilization in the later mentioned applications.

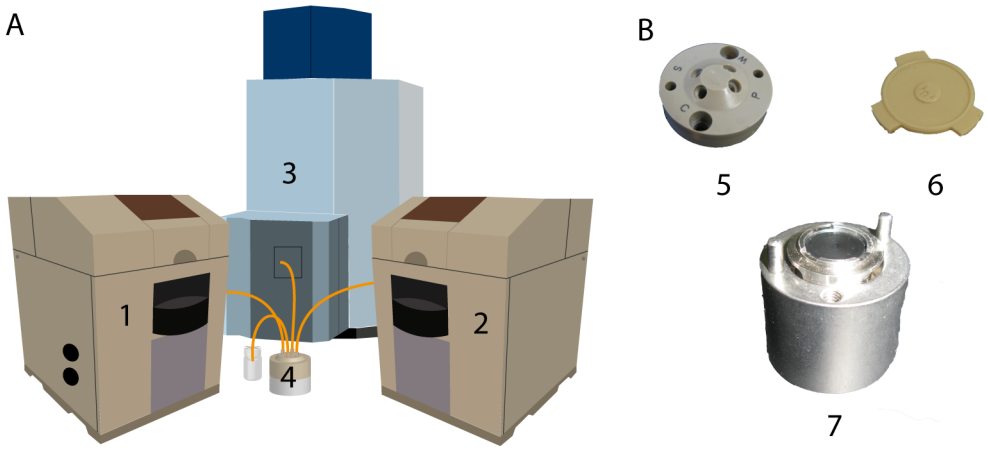

Figure 3.2: Arrangement of instruments (A) of CIEF(1)-CZE(2)-MS(3) using a mechanical valve (4) and valve in detail (B) including stator (5), rotor (6) and motor (7).

The entire instrument setup, including both valve positions is displayed in Figure 3.3. For the transfer of analytes from the first into the second dimension, the valve has to be in position A at the beginning of the experiment, positioning the sample loop in the first dimension. At the moment when the analyte is positioned in the loop, the first dimension is stopped and the valve is switched into position B. Thus, the large loop including the analyte is now in the second dimension (indicated in green). By switching on the voltage in the second dimension the analyte is migrating towards the ESI-MS detection, whilst separating from the remaining ampholytes.

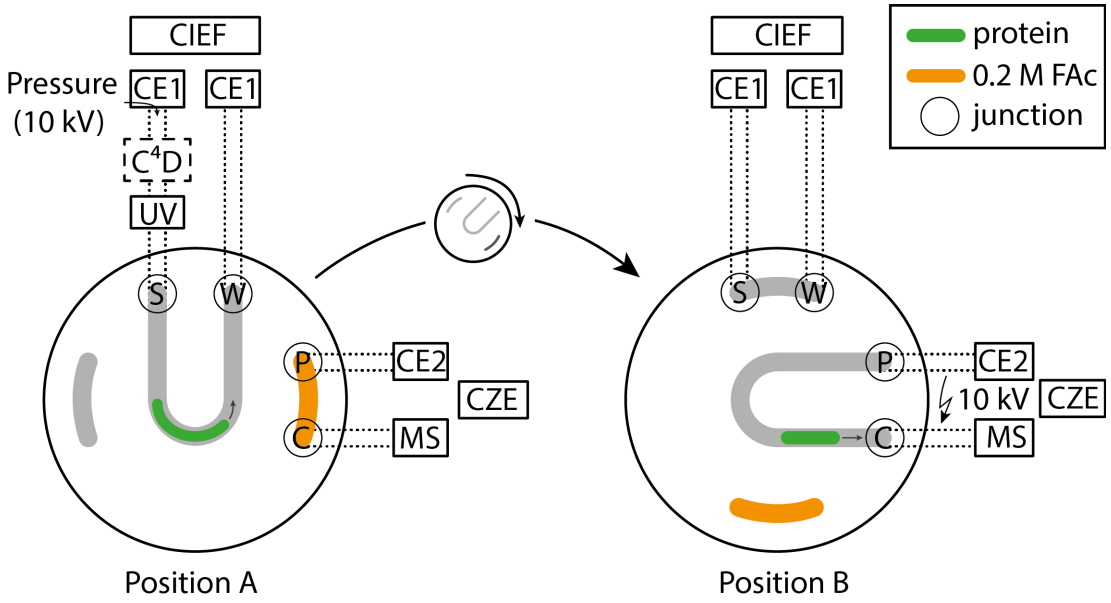

Figure 3.3: 2D electrophoresis instrument setup including load and inject position of the valve. The CIEF-(C4D)-UV- dimension is connected to junction S and W, while the CZE-MS dimension is equipped to junction P and C. Mobilization was performed with pressure and remaining high voltage. CZE was performed applying 10 kV for 20 min.

Placing spacer/stabilizer to focus the proteins before the UV detector

In general, there are two possibilities to detect isoelectrically focused proteins: (I) An imaging of the whole capillary or (II) a mobilization through/into a detection system. In the second case, all analytes have to be focused between the anolyte vial and the detector. In order to achieve this, the part between detector and catholyte vial has to be "blocked" by a substance with a high pI value, called catholyte spacer. The most common catholyte spacer is L-arginine. In most applications an additional anolyte spacer (iminodiacetic acid) is applied. The anolyte spacer is utilized to stabilize the CIEF system. Both, spacer or stabilizer, are added to the sample solution in an appropriate concentration mentioned in the corresponding article (**Paper II or III**).

Coating of the fused silica capillary

In order to prevent any adsorption effects of analytes e.g. proteins or peptides, the utilized fused silica capillary has to be neutrally coated. Different coatings were tested during the here presented work. In **Paper II** an Ultra Trol^{TM} Precoat LN coating was applied, which represents a physically adsorbed precoating. The coating has to be renewed prior to each analysis series (n$\leq$20), which results in a higher lab work and longer analysis time. Moreover, a slight electric osmotic flow (EOF) was determined, which results in an early mobilization during the focusing. This phenomenon can have an influence on the resolution. Therefore, for **Paper III** and **IV** a polyvinyl alcohol (PVA) neutral coating was utilized. The PVA coating represents a permanent chemical bonded coating, which is immobilized by polymerization. The coating procedure was performed inhouse and the capillaries were utilized for all applications in **Paper III** and **IV**.

Positioning of the analyte inside the sample loop of the valve

To detect and position analyte bands from the CIEF separation, an accurate detection system is necessary. A common type of detector for proteins and peptides is a UV absorption detector. An ideal 2D setup would enable the detection at the sample/transfer point. In the here presented case this is not possible due to the opacity of the valve material (PEEK/PTFE). The closest distance is approximately 4.5 cm in front of the valve. This allows the detection of the analytes but, nevertheless, requires to take the flow velocity into account for the positioning of the analytes. The relative positioning of the external detectors is also shown in Fig 3.3.

For a sufficient transfer of analyte from the CIEF into the CZE dimension a precise positioning of the analyte inside the sample loop of the valve is mandatory. A first methodology contains the calculation of the flow rate during the mobilization by taking the signal of the end of the L-arginine zone by applying conductivity and UV detection. Taking the flow rate, the distance between UV detector and valve and the UV mobilization time of the analyte of interest in consideration, it was possible to calculate the positioning time of the analyte inside the sample loop in a reproducible way. This calculation is described in detail in **Paper II** and was applied for all measurements in this publication. Nevertheless, the application of two external detectors in close distance in front of the valve is challenging, the detection of the end of the L-arginine zone can be difficult due to slightly differences in the signal intensity and a detection of two wavelength (200 nm for L-arginine and 280 nm for proteins) was necessary. For these reasons, for further measurements, the flow rate was determined prior to each run with an injection of 1000 mg/L caffeine. This allows the work without a second external detector and enables the optimization of the UV detector on only one wavelength. The reproducibility was comparable to the method described above. This second methodology was used for the measurements in **Paper III and IV**.

Evaluation of the CIEF-CZE-MS setup by the application of a model protein sample

In order to evaluate the above described CIEF-CZE-MS setup, an aqueous sample containing 40 ppm of three model proteins (ribonuclease A, myoglobin, β-lactoglobulin) was mixed with iminodiacetic acid as an anodic spacer, L-arginine as cathodic spacer and Servalyt^{TM} (3-10). As mentioned before, an Ultra Trol^{TM} Precoat LN coating was selected to prevent any adsorption of proteins at the capillary wall. The proteins were focused for 5 min with 10 kV and positioned individually into the valve. The UV signal of the mobilization by pressure and remaining high voltage can be seen exemplary in Figure 3.5. The positioning of the proteins inside the sample loop of the valve is explained in detail in **Paper II**. Once the protein of interest was positioned inside the loop, the mobilization was stopped and the valve was switched as illustrated in Figure 3.3. Due to the mean band volume of the focused proteins of 12 nL a sample loop of 20 nL was selected. 10 kV were used for the migration of the protein towards the ESI-MS instrument. The signal intensity at the migration time of β-lactoglobulin for the BPE at the MS detection was 2.26 x 10^4 $\pm$ 5.36 % (n=3). The high repeatability of the BPE intensity shows that the transfer of the protein from the CIEF separation into the CZE separation works precisely between different runs. For all three proteins, interference-free mass spectra were obtained (see also Fig 3.5). This is in good agreement with the separation of ampholyte and protein shown in Figure 3.1. The deconvoluted masses, displayed in Fig 3.5, from the proteins were in good agreement with the literature. By comparing the base peak intensities of the CIEF-CZE-MS approach with the CZE-MS, including an injection of 50 mbar for 24 seconds, a factor of 33 was determined. This is in good accordance to the estimated factor of 30 by comparing injection volumes.

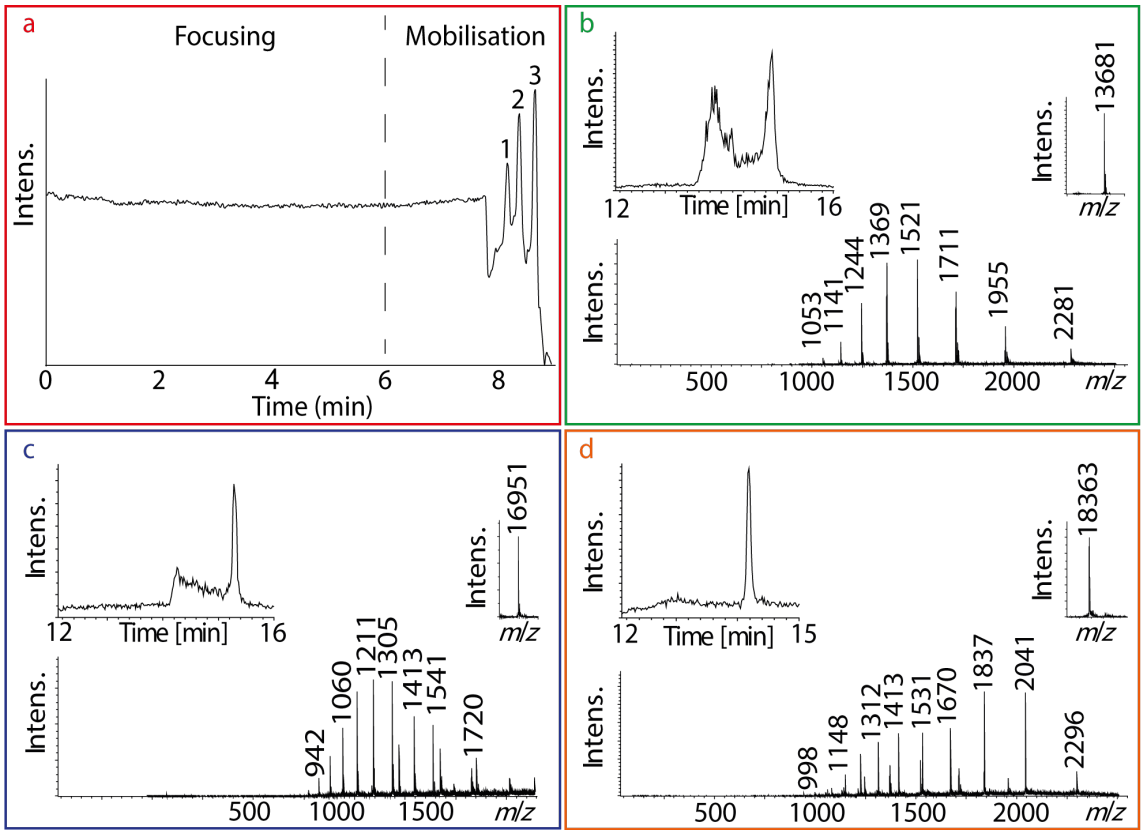

Figure 3.4: Electropherogram from the external UV detector at 200 nm (a). An abrupt fall of intensity indicates the end of the L-arginine zone (pI=11.2). Signals in a: ribonuclease A (pI=9.6) (1), myoglobin (pI=6.8-7.2) (2) and ß-lactoglobulin (pI=5.1) (3). Sample concentration 40 mg/L (protein). Focusing time: 6 min with 10 kV. Mobilization with 15 mbar and high voltage. Catholyte: 0.01 M H_3PO_4; Anolyte: 0.02 M NaOH. Second dimension with MS detection cutting the peak of ribonuclease A (1b), myoglobin (1c) and ß-lactoglobulin (1d), each with base peak electropherogram (upper left side), mass spectrum (lower part), and deconvoluted mass spectrum (maximum entropy, upper right side).

3 The MS analysis of CIEF separated hemoglobin and its glycated form

In order to demonstrate the power of the valve based CIEF-CZE-MS concept the separation of hemoglobin and the glycated form of hemoglobin was selected. These two analytes possess a similar structure and therefore, a pI difference of only 0.037 which are challenging to separate. The two analytes were detected at 420 nm in a reproducible way, however, not baseline resolved. The signals were individually transferred into the CZE-MS dimension where the ampholytes were separated from the protein. The transfer of signal 1 (Figure 3.5) resulted in a higher MS signal for the non-glycated form, whilst transferring signal 2 showed a higher signal intensity for the glycated hemoglobin. The incomplete individual cutting can be explained by the already overlapping signals in the CIEF mobilization, an excessive loop size inside the valve or a slightly incorrect cutting position. Nevertheless, this showcase demonstrates the possibility to cut and identify unknown signals of CIEF separated proteins by MS.

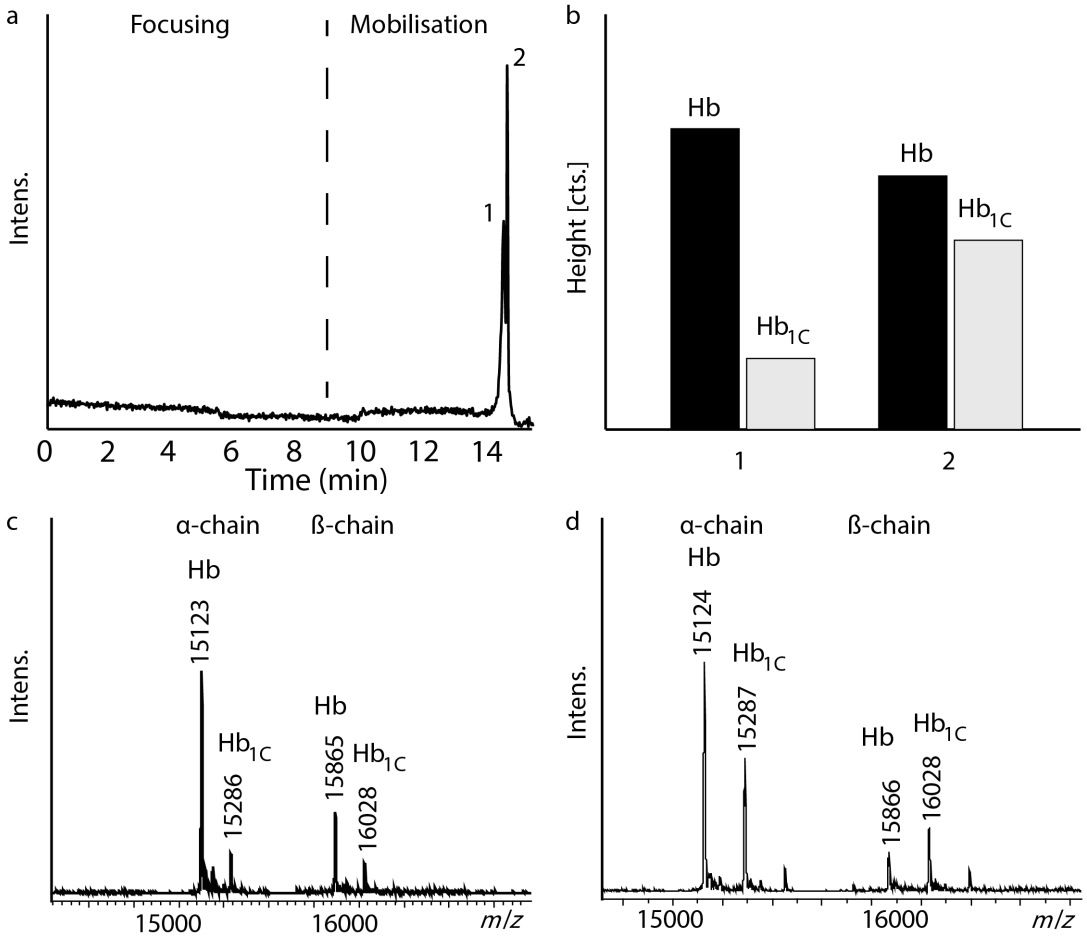

Figure 3.5: Electropherogram from the external UV detector at 420 nm (a). Focusing time: 9 min with 10 kV. Mobilization with 15 mbar and high voltage. Catholyte: 0.1 M H_3PO_4; Anolyte: NaOH 0.02 M. Ratio of hemoglobin and glycated hemoglobin by cutting on signal 1 and 2. Deconvoluted mass spectra (maximum entropy) of peak 1 (c) and peak 2 (d).

Compared with other CIEF-MS strategies, the here presented approach is a fast (30 min), interference-free and reproducible alternative even for structurally similar molecules. However, in further measurements different alterations at the procedure were tested e.g. regarding a faster, robust and more flexible setup. The successful improvements are described in the following section and were utilized for the following applications.

4 Improved sample positioning, multiple heart-cut and different transfer loop volumes for a wide range of model proteins

The transfer of the focused analytes towards the CZE-MS dimension is a crucial step in every multidimensional separation setup. As mentioned before in **Paper II**, the analyte positioning was performed with an external conductivity (C^4D) and UV detector. However, the positioning of two external detectors in close distance to the 4-port-valve is technically challenging. Therefore the positioning of the analyte in the sample loop for experiments of **Paper III** and **IV** was based on the application of an external UV detector and the determination of the flow rate prior to each run. The flow rate was determined with a caf-

feine standard (1000 mg/L) and 50 mbar. The detailed procedure is described in **Paper III** and **IV**.

In order to improve the transfer process, different sample/transfer volumes (20, 10 and 4 nL) were evaluated. The selection of the loop size should be performed based on the peak width and the resolution within the CIEF run. For instance, for the measurement of the wide range model proteins sample, a peak width of 0.09 (8 nL) to 0.13 min(11 nL) was calculated taking the flow rate and the UV electropherogram of the CIEF mobilization in consideration. The distance between the closest two signals was calculated to 0.13 min (11 nL). In this case, the 10 nL sample loop was found to be appropriate because the application of the 20 nL transfer loop lead to a transfer of more than one signal. Applying the 4 nL sample loop results in a low intense MS signal due to the transfer of only part of the focused analyte zone. The evaluation for the most suitable transfer volume was performed for all following applications and is described in the corresponding article (**Paper III** and **IV**).

In order to significantly reduce the overall analysis time combined with less sample consumption the previously utilized single heart-cut methodology was expanded to a multiple heart-cut approach. Therefore a standard mixture of five proteins and one peptide was focused, mobilized, transferred and analyzed in the CZE-MS dimension, consecutively. Each analyte could be identified by their respective MS spectra, as displayed in Figure 3.6. In this way, it was possible to transfer and analyze the proteins and peptide in a very reproducible way, covering a broad pI range (5.1-10.3). The results are shown in detail in **Paper III**).

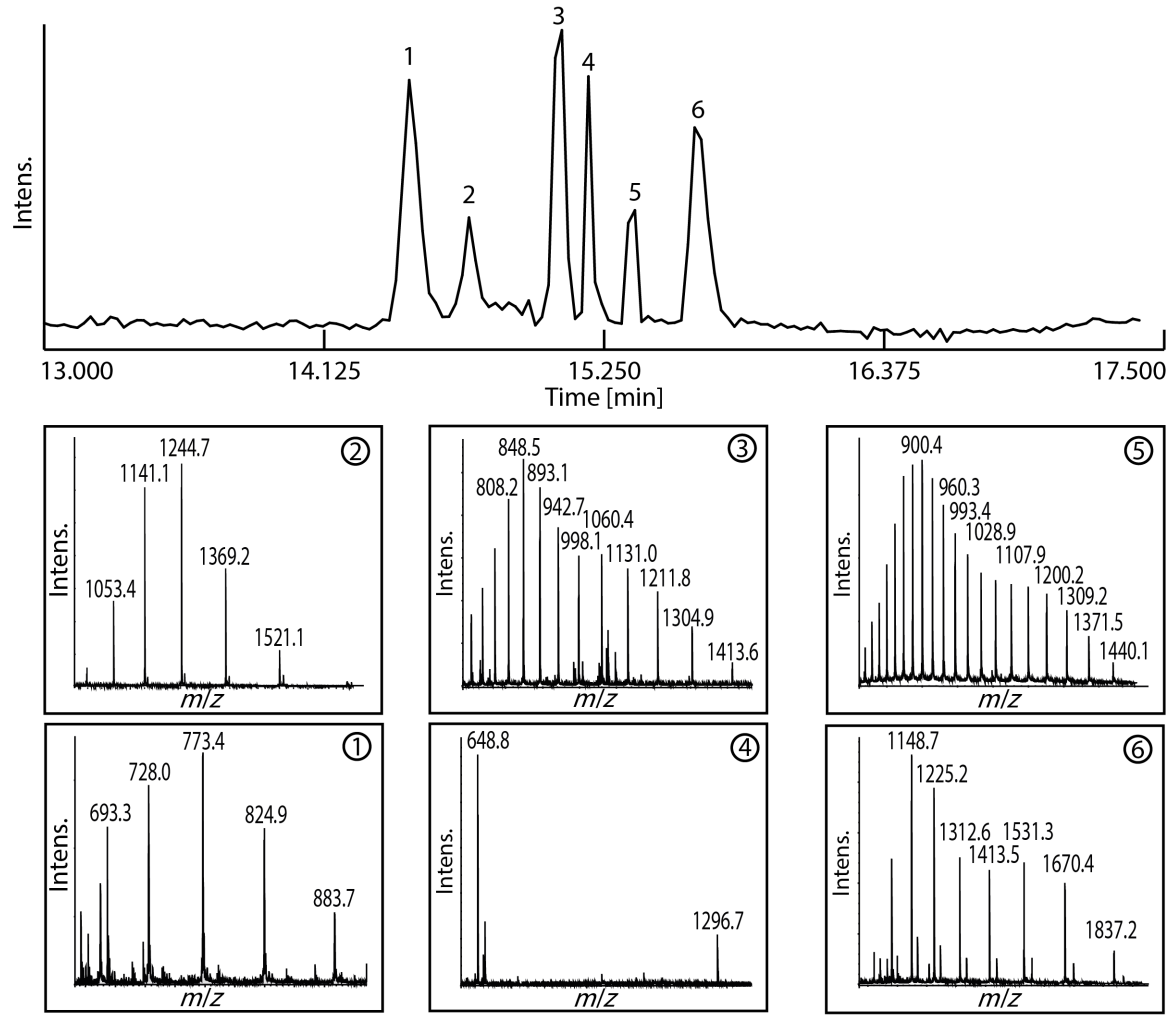

Figure 3.6: Mobilization of a focused (10 kV for 10 min) protein and peptide mixture applying 0.1 M H_3PO_4 as analyte and 0.2 M NaOH as catholyte. Mobilization was performed by utilizing 50 mbar (and remaining 10 kV) until the first signal is positioned in the 4-port valve. UV signal at 280 nm (main figure) and the mass spectra (inset 1-6) of all 6 transferred analytes: cytochrome C; pI 10.3 (1), RNaseA; pI = 9.6 (2), angiotensin I; pI = 7.9 (3), myoglobin; pI = 7.2 (4), carboanhydrase A; pI = 6.4 (5) and β-lactoglobulin; pI = 5.1 (6). Consequently, after CZE-MS analysis of the first analyte the valve was switched back to position A (Figure 3.3) and the next analyte was positioned in the sample loop by applying pressure (50 mbar). This procedure was repeated for all 6 analytes.

5 Multiple heart-cut CIEF-CZE-MS of mAb X

The separation of charge variants of mAbs represents a highly relevant application for CIEF, however, the detection via MS is challenging due to the ESI-interfering substances necessary for the CIEF process. Nevertheless, there is a high demand for MS characterization of mAb variants in the pharmaceutical industry. Therefore, the application of our 2D system for the analysis of mAb variants was evident. A deglycosilated mAb X sample (solved in formulation buffer) was injected and four signals could be detected in the CIEF dimension which is displayed in 3.7.
Signal 1 and 4 (Figure 3.7) were transferred and analyzed via CZE-MS. The raw and

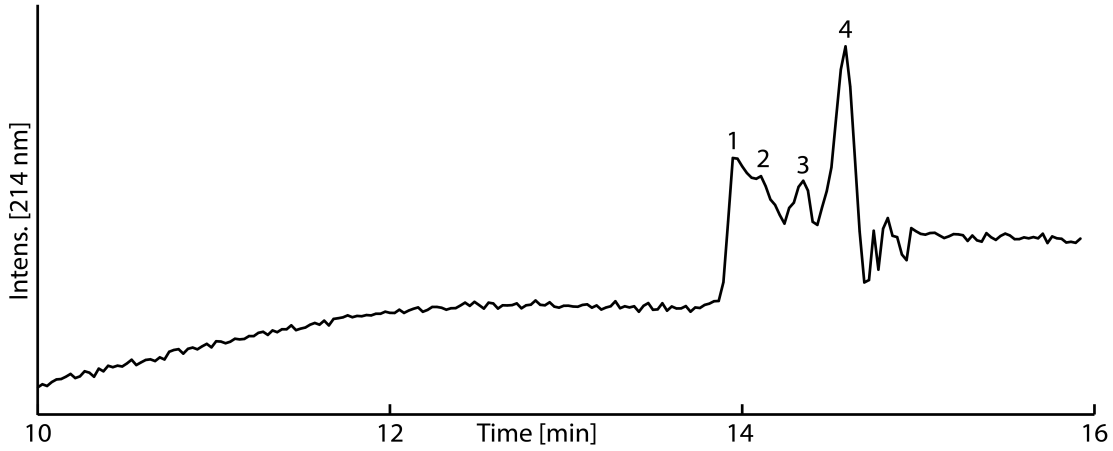

Figure 3.7: Presence of four different signals (signal 1-4) in the mobilization (50 mbar and remaining 10 kV) of deglycosilated mAb X. Focusing (10 kV for 10 min) was applied utilizing 0.2 M H_3PO_4 (anolyte) and 0.3 M NaOH (catholyte)

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deconvoluted mass spectra of both signals can be found in Figure 3.8. The deconvolution of signal 1 resulted in an exact mass of 145168.6 ± 0.4 Da (n=4). Signal 4 resulted in an exact mass of 145170.7 ± 0.6 Da (n = 4). A mass different of 2 Da could be detected, which can be potentially explained by double deamidation (or amidation). However, an interpretation of the relative intensity of the signals was not possible due to the occurrence of additional deamidation processes caused by the PNGase F treatment in the sample preparation [40]. Nevertheless, the separation and identification of variants of an intact antibody with a mass difference of only 2 Da indicates the power of this setup for the characterization of high mass proteins on intact level.

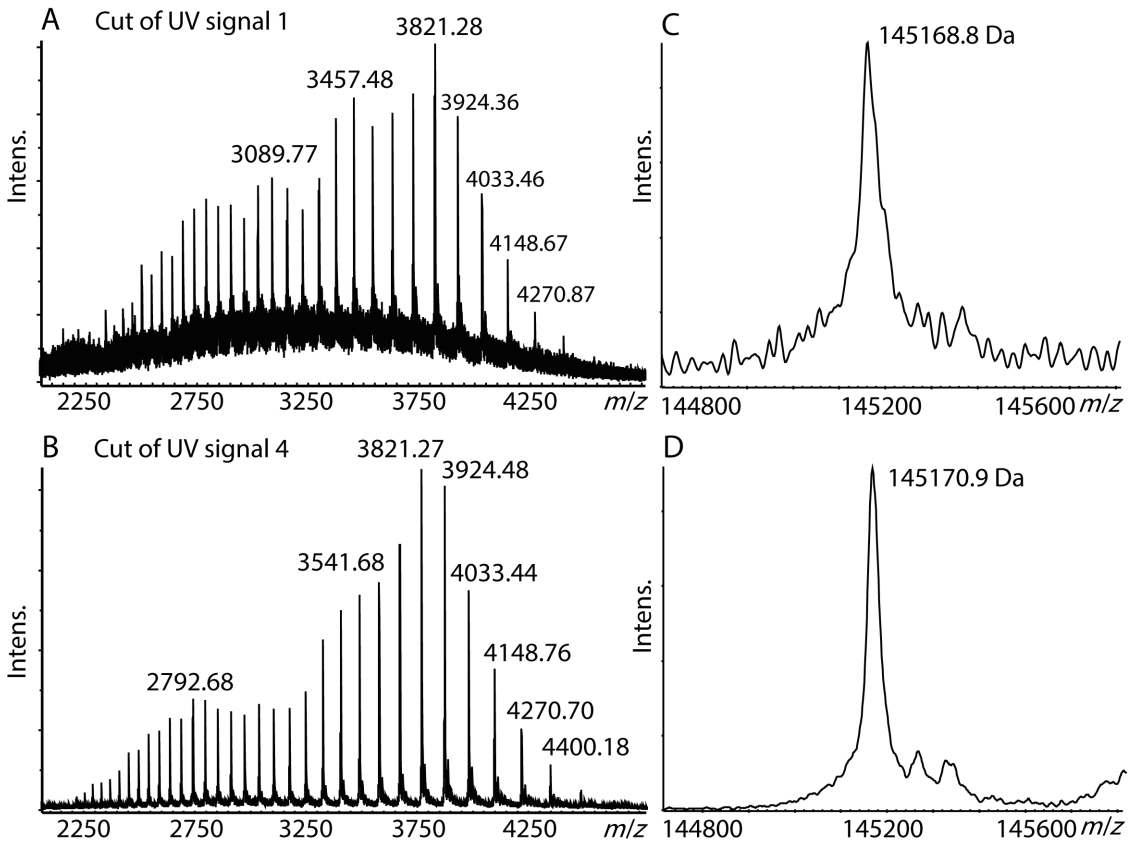

Figure 3.8: MS identification of mAb X variants from CIEF-CZE-MS separation. CZE-MS run of signal 1 (A) and 2 (B) and the corresponding deconvoluted mass spectra (C and D). A difference of 2 Da between the two variants was observed

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6 CIEF-CZE-MS - limit of detection for proteins

In general, CIEF provides a considerable enrichment factor in comparison to other electromigrative techniques, due to the IEF separation mechanism and thus, represents an excellent technique for the analysis of low concentrated samples. Therefore, the limit of detection (LOD) for low (10-50 kDa) and high mass (160 kDa) proteins was estimated. The LOD in the UV detection was calculated between 9.75 (RNaseA) and 1.63 mg/L (myoglobin). A lower concentration results in difficulties regarding the transfer of analytes in our 2D system. Nevertheless, the lowest concentrations lead to reproducible mass spectra with more than sufficient intensity. Thus, in the case of low mass analytes the UV detector represents the limiting factor for the 2D setup. Moreover a more sensitive external detection would enable an even lower overall LOD. In the case of large mass protein samples, e.g model antibody mAb X, the MS showed to be the limiting factor regarding sensitivity. For accurate deconvolution, a concentration of 1500 mg/L of mAb X was required. Nevertheless, appropriate mass spectra for the main variant were achieved applying a mAb X concentration of 375 mg/L (no accurate deconvolution possible). The rather high LOD of 1500 mg/L for mAb X can be explained by the low ionization efficiency of high molecular weight species. Nevertheless, this represents an excellent value compared to previous work regarding CZE-MS: Biacci and coworkers demonstrated in 2015 a glycoform separation and characterization by offline coupling of for instance CZE-UV with ESI-MS with a sample concentration of 5000 mg/L [41]. Another common top-down approach for the analysis of

large mass proteins is provided by UHPLC-MS. Applying this approach, Liu et al., were analyzing free thiol variants of a mAb utilizing a concentration of 2000 mg/L. An in-house comparison between our CIEF-CZE-MS setup including a 10 nL (sample concentration of 1 500 mg/L mAb X) and a CZE-MS analysis applying an injection volume of 10 nL (sample concentration of 30 000 mg/L mAb X) was performed. By taking the different concentrations into consideration an enrichment factor of 23 for the CIEF-CZE-MS system was found.

7 Characterization of model mAb by CZE-CZE-MS

As demonstrated before, CIEF represents an excellent technique for the separation of mAb variants, nevertheless, a common alternative is a CZE separation applying an ϵ-aminocaproic acid (EACA) based electrolyte system (0.380 M EACA, 0.002 M triethylenetetramine (TETA), 0.05% w/w HPMC, adjusted with acetic acid (HAc) to pH 5.7) [42][43]. This approach was recently utilized in an inter-laboratory study for the analysis of 23 mAbs showing impressive precision and accuracy [44]. For the characterization of the separated variants, MS detection is mandatory. Unfortunately, the aforementioned EACA electrolyte system is highly ESI-interfering. Thus, the same challenge arises as previously stated with the CIEF to MS hyphenation: A frequently applied separation dimension consists of an ESI-interfering compound. Nevertheless, its again possible to apply CZE-MS, utilizing a 2 M HAc BGE, as a second dimension, to separate the ESI-interfering electrolyte components and the mAb sample band and enable an interference-free MS detection. The general instrument setup, the positioning and transfer of the sample as well as the detection is similar to the CIEF-CZE-MS system and extensively explained in **Paper IV**. Figure 3.9 displays the variant separation of an 18.000 mg/L mAb X sample applying the aforementioned EACA buffer system. The raw as well as the deconvoluted mass spectra of the transfer of the main variant (signal 2 in Figure 3.9) into the CZE-MS dimension shows the typical glycoform pattern of mAb X. For the highest abundant glycoforms G0F/G0F, G0F/G1F and G1F/G1 (G0F/G2F) an average mass of 148059.1 ± 0.8; 148220.1 ± 0.5 and 148381.6 ± 0.6 (n=5) was determined, respectively.
In general, basic and acid variants of mAbs are considerably less concentrated compared to the main form. Thus, the influence of concentration on the mass accuracy was tested. The results, which can be found in **Paper IV** indicate no significant influence on the detected masses. Nevertheless, the analysis of the acid variant (signal A2 in Figure 3.9) with the CZE-CZE-MS system results in a very noisy MS signal and thus, not sufficient for repeatable accurate mass determination. In order to increase the sensitivity and simultaneously simplify the spectrum, a deglycosilation of the mAb sample was performed using N-glycosidase F. The detailed protocol can be found in **Paper III** or **IV**. Utilizing this deglycosilated sample the main variant (signal 1) and the two highest acidic variants (signal A2 and A3) were analyzed. The mass difference between the acidic variant A2, A3 and the main variant was + 2.0 Da and + 3.1 Da, respectively. As also stated by the CIEF-CZE-MS analysis of the model mAb, a potential explanation is the presence of deamidation products. Thus, the acidic variants A2 and A3 could correspond to a double and triple deamidation, respectively.

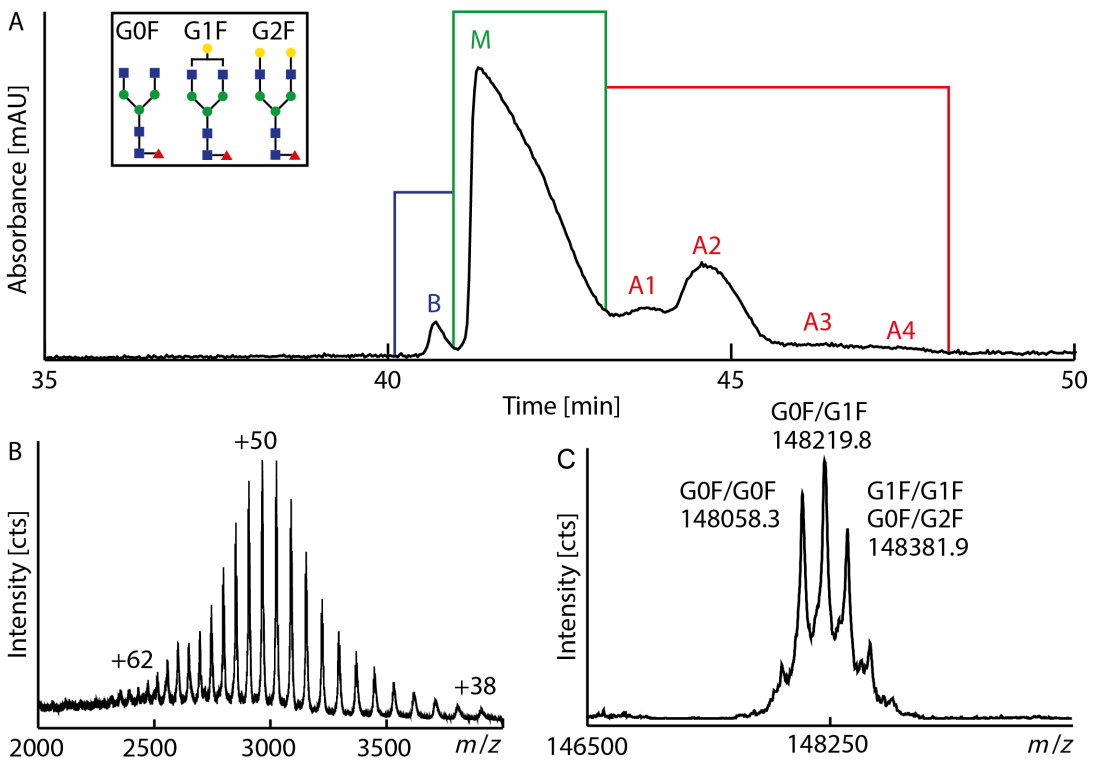
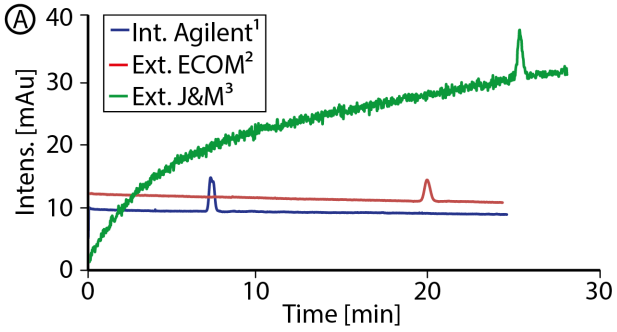


Figure 3.9: A: UV signal at 214 nm of separated variants of a model mAb X in an EACA based BGE (0.38 M EACA, 0.0019 M TETA and 0.05% w/w (hydroxypropyl) methyl cellulose (HPMC) (pH = 5.7, adjusted with HAc; 10 kV for 60 min). B and C: Raw and deconvoluted mass spectra of cutting the main variant of the model mAb. B: charge states range from +38 to +62 in the raw mass spectra. C: the three highest signals were assigned to the corresponding glycoforms.

8 Comparison of different external UV detectors

An important part of the here presented 2D setup is represented by the detection in the first dimension. Transfer of analytes into the second dimension is only possible if the analyte is clearly identified by its UV signal. An insufficient UV intensity lead to a higher overall LOD. All experiments covered by **Paper II to IV** were performed with the Tidas S700 external UV detector from J&M (Essingen, Germany) [later referred to: J&M det.]. This detector was already in the laboratory at the beginning of this work. In course of the project, a second external UV detector (ECD26000) was purchased from ECOM (Prague, Czech Republic) [later referred to: ECOM det.]. Both external detectors were directly compared with focus on signal to noise values and were set in comparison to the internal UV detection applying a HP ^{3D}CE electrophoresis instrument from Agilent (Agilent Technologies, Waldbronn, Germany) [later referred to: int. Agilent]. Both external detectors are applying an optical cell, where the fused silica capillary is fixed in. The optical cell is furthermore connected to the lamp/detector via optical fibers. To test the signal to noise value of all three detection units, a fused silica capillary was equipped to an Agilent ^{3D}CE instrument. The capillary was filled with 0.2 M FAc. Afterwards a solution of 1 mg/L caffeine in water was injected by applying 50 mbar for 24 sec. 30 kV were utilized for electrophoretic mobilization. The three detection systems, int. Agilent, ECOM det. and J&M det., were positioned in this sequence at the capillary and UV detection was applied

with 200 nm. The electropherograms of all three detectors are displayed in Figure 3.10A. A relative high noise can be noticed by comparing the external UV detector from J&M to the other two detection systems (see also Figure 3.10). A similar noise were found by comparing the external UV detector from ECOM and the internal UV detection (Agilent). The lower signal applying the ECOM detector in comparison to the internal UV detection can be explained by the utilization of the optical fibers (50 cm before and 50 cm after the optical cell) which lowers the overall intensity. The optical fibers are not present with the internal UV detection.



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UV detector	Noise [mAu]	Signal [mAu]	Signal to noise
Int. Agilent[1]	0.05	10.5	210
Ext. ECOM[2]	0.05	4.5	90
Ext. J&M[3]	2	7.5	3.8

Figure 3.10: Direct comparison of two different external UV detectors and relative comparison to the internal UV detector (Agilent CE). A: electropherograms with 200 nm of 1 mg/L caffeine applying 0.2 M FAc and 30 kV utilizing all three detection systems simultaneously. B: Comparison of noise, signal and signal to noise values for all tested detectors. [1]HP ^{3D}CE electrophoresis instrument from Agilent; [2]ECD2600 from ECOM; [3]Tidas S700 from J&M.

Afterwards, a second experiment was performed to differentiate between detection unit and optical cell concerning noise. Thus, the optical cell, purchased from ECOM was equipped to the detection unit received from J&M. A sample containing 50 mg/L myoglobin in water was injected by applying 50 mbar for 24 sec. Same electrolyte and voltage were applied as stated in the experiment explained above. All received electropherograms including the corresponding detector and optical cell arrangement are displayed in figure 3.11.

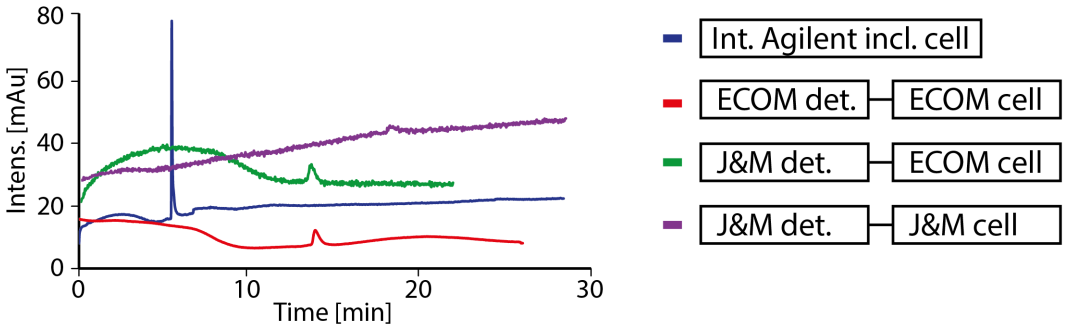

Figure 3.11: Comparison of different detector/optical cell arrangements in relative comparison to the internal UV detector (Agilent). Electropherograms with 200 nm were received by injecting a sample of 50 mg/L hemoglobin in water and applying 0.2 M HAc as BGE and 30 kV as mobilization voltage.

As indicated in Figure 3.11, by utilizing the J&M detector, a higher noise is received. This is similar for both optical cells. Thus, the noise is mainly produced by the detection unit and not by the optical cell. Moreover a slightly smaller signal was detected by applying the

J&M detector with the optical cell from J&M. In summery, the optical detection system which was purchased from ECOM shows a factor of 30 higher signal to noise level which is a big advantage for all future experiments, utilizing an external UV detection. Thus, the ECOM external UV detector was applied for all future experiments e.g. for the nano LC-(UV)-CZE-ESI-MS coupling which is described in the next section.

9 Nano LC-(UV)-CZE-ESI-MS for proteins and their corresponding variants

As previously mentioned, the separation and characterization of protein variants is of high demand. A system of high potential could be the online combination of nano LC and CZE-MS. Nano LC provides, compared to CZE or CIEF, high injection volumes and robust separation of proteins, whereas CZE-MS is perfectly suited for the separation and characterization of protein variants. A concept of such a nano LC-CZE-MS system separating proteins in first and their corresponding variants in a second dimension is displayed in Figure 3.12.

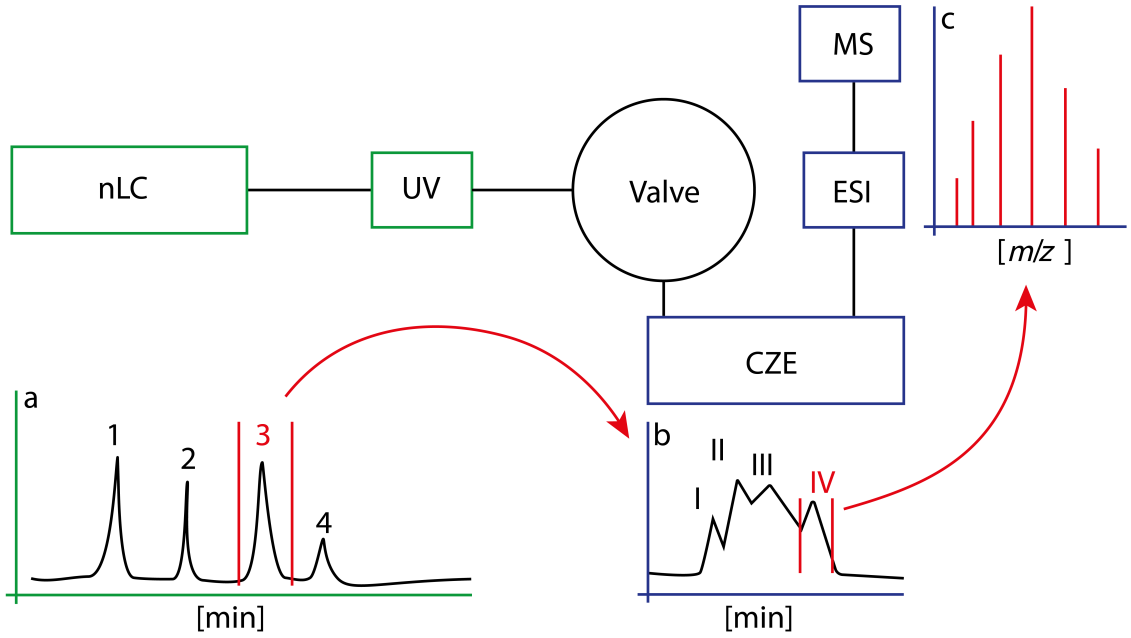

Figure 3.12: Concept of a nano LC-CZE-MS setup for the separation of proteins (a), followed by their corresponding variants (b) including identification via MS (c).

As stated before, the hyphenation device is the crucial part of every multiple dimensional system. Including a CE dimension in such a system requires a fully electrically isolated transfer device, including zero dead volume connections. The 4-port-valve which was applied for the CE-CE-MS systems, intensively presented in the last chapters, was promising to achieve a nano LC - CZE coupling. Hence, a nanoFlow 1200 pump from Agilent, Technologies (Waldbronn, Germany) including a degasser unit was connected to the 6-port-valve from a *compact* QqTOF instrument from Bruker Daltonik (Bremen, Germany). The 6-port valve, including a 250 nL sample loop, was used for injecting the sample. The full setup can be seen in Figure 3.13. The LC separation was applied on a Zorbax 300SB-C8, 0.075 x 50 mm 3.5 micron column from Agilent Technologies (Waldbronn, Germany). Transfer of sample into the CZE-MS dimension was performed as explained in the CE-CE

hyphenation chapters utilizing the 4-port-valve or in **Paper III**. Both, the nano LC-UV and the CZE-MS, were tested individually prior to the complete 2D setup.

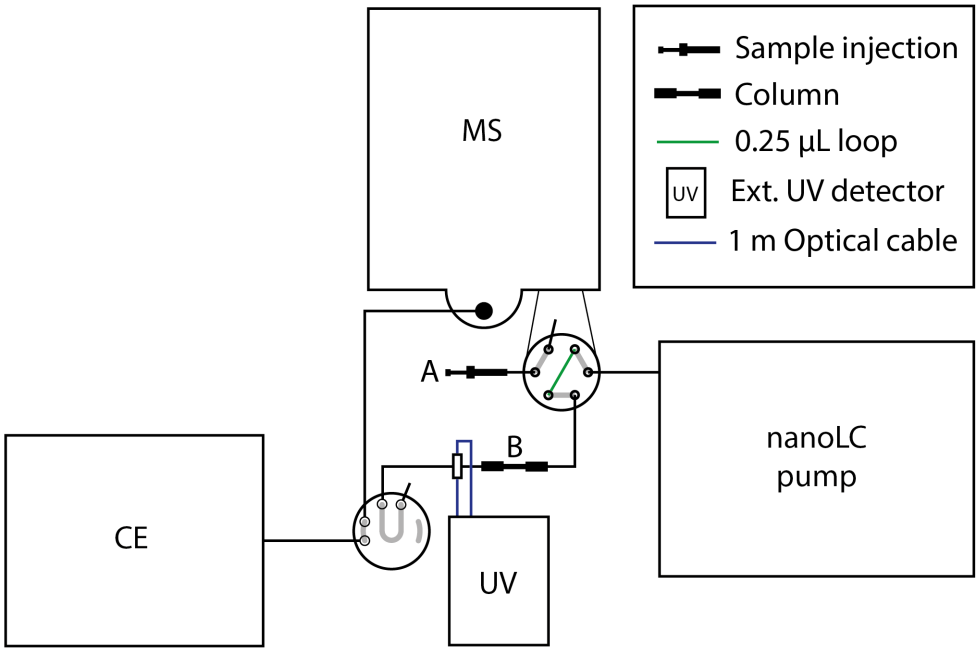

Figure 3.13: Setup of the nano LC-CZE-MS setup including nano LC pump, 6-port injection valve including a 100 µL syringe (A) to fill the 250 nL sample loop, Zorbax 300SB-C8 capillary column (B), external UV detection (ECD2600 EX UV - VIS, ECOM spol. s r.o., Prague, Czech Repuplic), the electric isolated 4-port-valve for sample transfer, the CE and MS instrument.

The first glycoprotein variant separation applying CZE-MS was performed by Kelly and coworkers in 1996 [45] and further developed by e.g. Sanz-Nebot et al. [46] for transferin variants and Balaguer et al. [47] for variant separation of erythropoietin (EPO), fetuin and 1-α-glycoprotein (AGP). The commonly applied formic (0.25-2 M) and acetic acid (1-5 M) BGEs were applied in different concentrations for the variant separation of AGP (human and bovine), transferin, EPO, fetuin and RNase B. The best separation in an appropriate time was found by utilizing 0.5 M formic acid and 15 kV in 70 cm PVA coated capillary. The PVA coating procedure can be found in **Paper III**. The mobile phase for the nano LC separation of the proteins was composed of A (H_2O+0.1 % acetic acid + 0.1 % trifluoracetic acid) and B (acetonitril + 0.1 % acetic acid + 0.1 % trifluoracetic acid) applied in gradient mode (25-85 % B in 20 min; flow rate: 0.5 μL/min) [48]. The ESI-MS parameter of the 2D experiments were similar to the CIEF-CZE-MS measurements described in **Paper III**.
A first nano LC-(UV)-CZE-MS experiment was performed analyzing a standard protein mix containing RNase A and B, cytochrome C, AGP (bovine) and myoglobin (50 mg/l in water each). The UV signal of the chromatogram can be seen in Figure 3.14 A. Consequently, signal 1 which corresponds to RNaseA/B was transferred via 4-port-valve into the CZE-MS dimension. The electropherogram of the CZE-MS dimension is displayed in Figure 3.14.

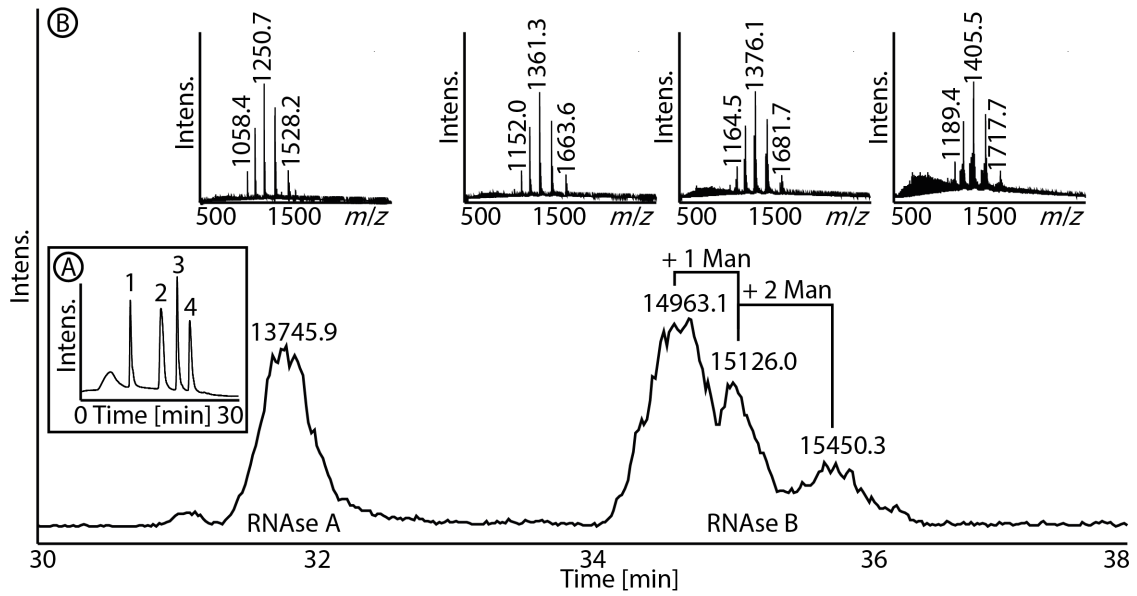

Figure 3.14: Nano LC-CZE-MS of model proteins. A: UV chromatogram of the separation of RNase A and B (1), cytochrome C (2), AGP (3) and myoglobin (4). B: Base peak electropherogram and the MS spectra of the CZE-MS dimension (BGE: 0.5 FAc; 15 kV for 40 min). A separation of RNaseA and B (including variants) was achieved in the second dimension.

Baseline separation between RNase A and B was achieved in the CZE-MS dimension. Moreover, it was possible to detect different high mannose glycoforms of RNase B. This first proof of concept measurement takes the advantage of the high orthogonality of CZE and LC and shows the power of this combination. Up to this point, CZE-MS experiments for the determination of charge variants of low mass proteins were performed analyzing sample concentrations of 1000-5000 mg/L [46][47]. Here, the experiments were performed with 50 mg/L, which represents a factor of 20-100 lower concentration. Moreover the possibility to characterize the variants of different proteins in a mixture is given. This represents an unique feature of the nano LC-CZE-MS system in comparison with CZE-MS where overlapping and interfering of different protein variant signals would be a common issue.

10 Alternative interface for the coupling of electrodriven or microfluidic separation techniques

The crucial part of a multiple dimensional setup including an electrically driven separation represents the coupling device. There is a need for continuous improvement and development of 2D coupling devices. During the work with the valve interface ,which was utilized for all previous measurements, requirements for a new interface were defined: i) It should be possible to work with materials which possess a high dielectric strength e.g. fused silica. ii) The new interface should provide straight channels to provide a homogeneous electric field and liquid flow. iii) The distance between the channels should be enough to prevent current breakthroughs or liquid transfer. iv) Detection at the transfer position should be possible. v) The interface device should be easy to fabricate. Keeping this thoughts in mind, a concept for an innovative chip-based valve was worked out. The design of the new interface can be seen in Figure 3.15. It consists of three parts which are cut out from one manufactured chip. The horizontal channels were produced prior to cutting to create a

smooth surface between the chip parts.
For a 2D approach separation dimension 1 (D1) is connected to the middle channel and separation dimension 2 (D2-MS) is attached to the lower channel (3.15 B). The separation in dimension 1 is stopped at the time when analyte three is reaching the middle chip part (3.15 C). The middle part of the chip, also referred to as the bar, is moved down to transfer analyte 3 into separation dimension 2 (Figure 3.15 D to E). Afterwards the separation in dimension 2 can be initiated.

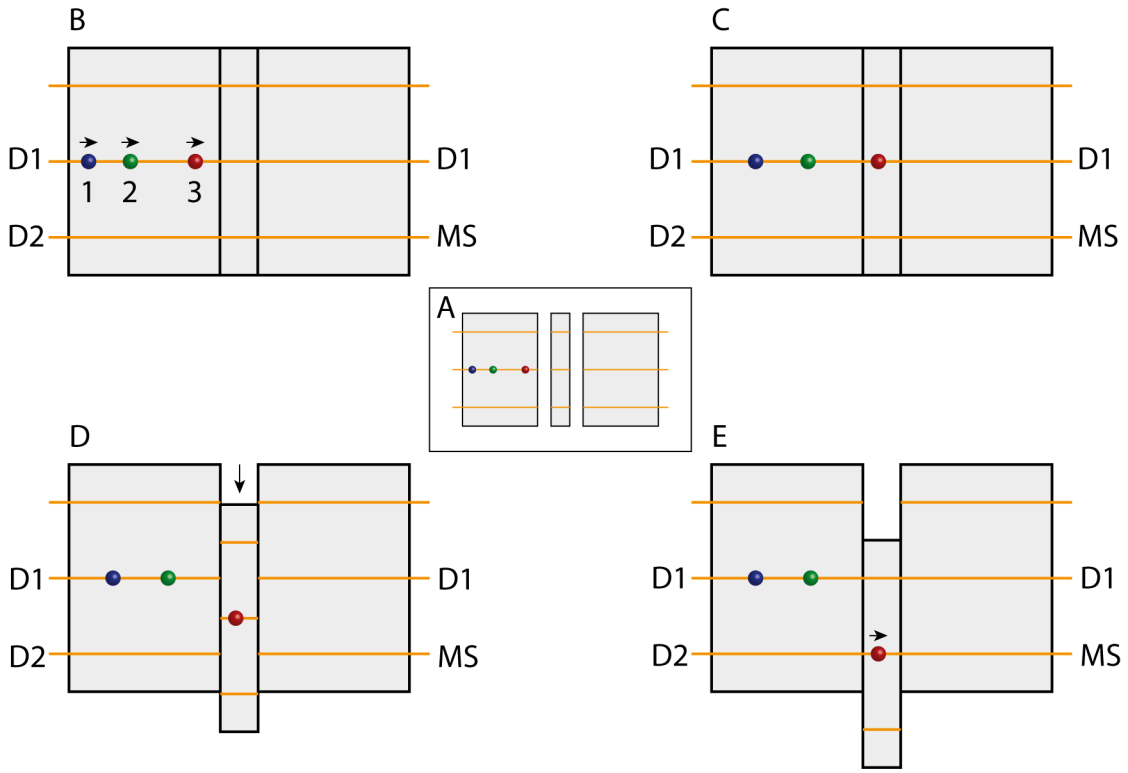

Figure 3.15: Principle setup and work flow of the chip-based interface. The interface consists of three chip parts (A) cut from one manufactured microfluidic chip. For the hyphenation of two separation dimensions the three parts of the chip are carefully pushed together. The transfer of an analyte (1-3) from separation dimension 1 (D1) to the separation dimension 2 (D2-MS) is displayed in Figure 3.15 B-E: Separation 1 (B) is stopped if analyte 3 reaches the middle chip part (C). The middle part of the chip is moved down (D) until the analyte on the middle part of the chip reach the separation dimension 2 (E).

For the realization of this chip based interface the mounting of the whole interface and the movement of the bar was important. The mounting should enable the fixation of the outer part of the chip, while the middle part is still movable. Therefore, the pressure on the outer part has to be adjustable. A combination of plates, brackets and micrometer screws was first simulated and subsequently constructed with help from the university machine shop. The simulation and the final construction can be seen in Figure 3.16.

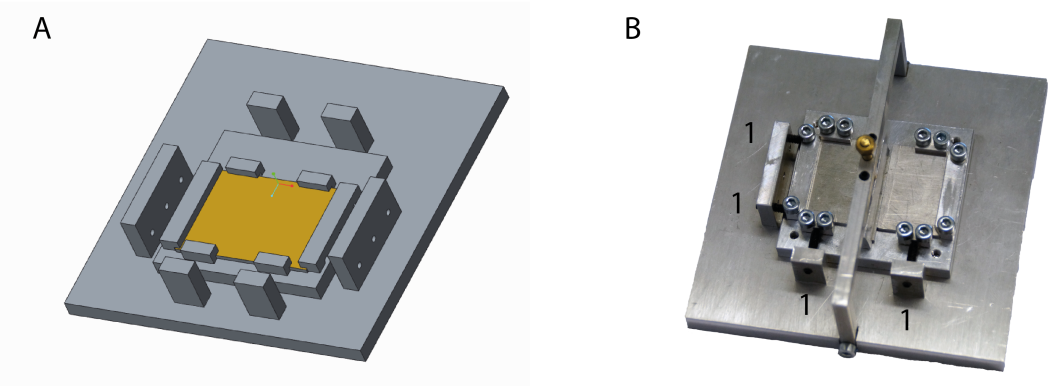

Figure 3.16: Simulation (A) and picture (B) of the brackets and screws for the fixation of the chip based interface (yellow square in A; transparent chip in B). Screws (1) to adjust the pressure on the three parts of the chip)

For the transfer of the analytes between the separation dimension, a movement of the bar is mandatory. This mounting and the bar is hold in horizontal position by ball-bearing suspension mounted above. The movement of the bar is performed manually which seems to be sufficient for tests in this early state of development. Fused silica capillaries, necessary for the separation dimension were glued to the end of the chip channels. In order to avoid on one hand dead volume and on the other hand blockage of the channel, the glue has to be cured short before entering the separation channel on the chip. After several tests with different kind of glues, a glue which can be cured by UV light (Bluefixx reparation pen, Westfalia Werkzeugcompany, Hagen, Germany) was chosen. After the fixation of the capillaries a first proof of concept test, regarding tightness of the three chip parts, was performed. After the installation of this prototype in the lab and setting up the next necessary steps the project was handed over to another PhD student for further development.

4 Conclusion

Multidimensional separation including CZE-MS as second dimension are perfectly suited for the separation, identification and characterization of proteins on intact level covering a wide mass range. A crucial part of such an electromigrative driven multidimensional setup is represented by the applied hyphenation interface. The here utilized and discussed 4-port-valve provides fully electrically divided separation dimensions, connections with low dead and suitable transfer volume. However, a limitation of 18 kV separation voltage is associated with the applied valve material and the close distance of the channels.
Nevertheless, the current 4-port-valve was utilized for impressive applications such the MS identification of CIEF separated hemoglobin and the glycated form and the characterization of deamidation variants of a monoclonal antibody. These variants were separated in a first approach by CIEF and in second by CZE in the commonly applied EACA based BGE. Both separations were online hyphenated to CZE-MS by the aforementioned 4-port-valve. These two applications represent the first online MS characterization of mAb deamidation variants and can play an important role in the biopharmaceutical field concerning e.g. stability and release testing.
The here presented hyphenation device allows besides the aforementioned CE-CE-MS couplings, also the hyphenation of the disseminated HPLC to CZE-MS. Two separation mechanisms which act almost orthogonal. A first proof of concept measurement, showing the separation of proteins and the corresponding variants at low concentration levels, indicates that this concept can act as a protein variant expansion to the commonly applied nano LC-MS top-down proteomics approach.
The above mentioned limitations were the reason for the development of a new chip based fused silica interface, which is expected to be utilized in future CE-CE or LC-CE applications. It provides straight channels, no voltage limitations, flexible transfer volumes and the possibility for detection at the sample transfer point.
In conclusion, CE will play an important role regarding the characterization and identification of proteins on intact level when it is hyphenated to other separation techniques. These multidimensional setups enable interference free MS detection, in the case of CIEF and EACA based CZE utilized as first dimension, or allow large injection volume and a preseparation, by applying LC as first dimension.

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List of Figures

5 Appendix

Paper I

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Received April 9, 2015
Revised July 31, 2015
Accepted July 31, 2015

Review

Capillary isoelectric focusing-mass spectrometry: Coupling strategies and applications

IEF on immobilized pH gradient strips is a widespread tool for protein separation, especially as first dimension in commonly utilized 2DE. In the latter arrangement, separations are based on two orthogonal molecular characteristics according to p*I* in the first and molecular weight in the second dimension. However, the approach is time consuming, quantification is difficult and MS can be applied only offline. Capillary IEF and related IEF techniques in combination with MS provide similar information. The major benefits are high mass resolution and mass accuracy, reproducibility, speed, automation, and quantification by using a high-resolution mass spectrometer. However, online hyphenation of CIEF with MS is interfered by the ampholytes, acids, and bases needed for high-resolution IEF. This review will give an overview about important coupling techniques, like low ampholyte concentration, interim separation by chromatography, or the use of a dialysis interface to separate the analytes from interfering substances. It is focused on strategies which allow sensitive MS detection of CIEF-separated analytes. In addition, proteomic and biopharmaceutical applications of capillary IEF techniques combined with MS are briefly summarized.

Keywords:
IEF / Monoclonal antibody / MS / Online hyphenation / Peptide / Protein
DOI 10.1002/elps.201500185

1 Introduction

It is well known among separation scientists and researchers in proteomics that IEF is a highly powerful strategy to separate amphoteric molecules in accordance to their p*I* into narrow zones along a pH gradient under influence of an external electric field. The molecules migrate through the electric field, as long as they possess a charge. Reaching the position where the pH equals the p*I* of the molecule, the net charge of the molecules becomes zero and they get focused in this region. IEF can be carried out in gels or solutions with mobile or immobilized pH gradients.

IEF is of central practical relevance for the separation of proteins and peptides in complex biological samples. It has recently also regained increasing attention for the analysis of charge variants of biopharmaceuticals (i.e., protein therapeutics such as antibodies) [1–3]. IEF is of particular interest in such separations because it provides besides efficient separation of (sometimes structurally closely related) proteins with the determined p*I* also useful molecular information for its characterization.

In general, IEF can be performed in a flat gel, in a "Rotofor", in a column or in a capillary (CIEF) [4]. Especially the capillary-based systems can further be distinguished into systems without ampholytes, with ampholytes, and such with ampholytes immobilized on polymeric supports, e.g. on an organic polymer monolith [5]. The most common kind of CIEF is achieved by applying voltage to an ampholyte system inside the capillary to create a continuous pH gradient [4]. The whole process of CIEF can be divided into two steps. First, the focusing step with an acid at the inlet and a base at the outlet vial and as a second step, the mobilization of the analytes to the detection system. General advantages of CIEF compared to flat-based IEF systems are the higher throughput, the easier handling, the opportunity of automation, and the better quantification possibility.

In its most common application, IEF on IPG strips is combined with SDS-PAGE to get a 2D system, which provides information about the p*I* value and the mass of the analyte [3]. For a long time, it has been the state-of-art separation technology in proteomics using a mass spectrometer for offline structural characterization and identification via subsequent database search [6]. Manual handling steps and long run times (for 2D gels) increasingly leads to its

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Abbreviations: FFE, Free flow electrophoresis; **FTICR**, Fourier transform ion cyclotron resonance; **UV**, Ultra violet

Colour Online: See the article online to view Figs. 5–7 in colour

replacement by other methods (primarily nanoLC-ESI-MS/MS of tryptic digests of the whole proteome–shotgun proteomics [7]). Attempts to replace nanoLC as separation technique by complementary and more efficient CIEF in online hyphenation with MS were hampered in the past by some practical complications such as interferences with the ionization process, commonly ESI. Ampholytes from the CIEF separation step may enter into the ion-source which results in electrospray instabilities, signal fluctuations, salt deposition in the ionization interface, and ion suppression [8]. It has been reported that the addition of 2% (v/v) carrier ampholytes to the BGE can lead to 45% signal loss [9]. Furthermore, commonly employed acids (phosphoric acid) and bases (NaOH) in inlet and outlet vials used to generate the pH gradient are nonvolatile BGE constituents as well, and likewise contribute to electrospray stability problems in the ion source and ionization process, respectively [8]. Thus, users of CIEF-MS run into problems because high ampholyte concentrations are necessary to achieve high separation efficiencies. Therefore, a compromise must be accepted between separation efficiency of the CIEF step and sensitivity of MS detection, when directly hyphenating both techniques [9]. In order to circumvent this problem, different approaches have been pursued. For example, MALDI has been adopted as an alternative ionization technique, immobilized pH gradients, or separation of the ampholytes from the sample before their mobilization into the MS ion-source employing for instance a dialysis interface or an intermediate liquid chromatographic separation step for the removal of the ampholyte from the sample zones.

CIEF with ultra violet (UV) detection is currently still state-of-art in the field but it is evident that online hyphenation with MS would have significant advantages if it is robust, straightforward, and mature. Narrow peaks require (preferentially) fast scanning instruments like (Q)TOF-MS and such MS instruments exhibit excellent mass resolution and high mass accuracy. ESI yields multiply charged protein species which can be detected as charge envelope from which the protein mass can be reconstituted by simple calculations. This may allow their straightforward identification. To some extent, even co-migrated protein species can be identified because they typically give superimposed but resolved charge envelopes in the mass spectra and additional molecule peaks in the deconvoluted spectra. Furthermore, it is possible to identify unknown signals by subsequent database search, if available. Unfortunately, CIEF-MS has some limitations as: (i) A connection between the CIEF capillary and the interface or directly the electrospray has to be realized. (ii) The electrode for the focusing process has to be applied between the CIEF and the MS system. (iii) The entering of electrospray disturbing substances like ampholytes or strong nonvolatile acids and bases from electrolyte vessels has to be prevented. Attempts to overcome these limitations, unfortunately, always result in more complex coupling interfaces than a simple optical detection. In principle, CIEF-MS may exhibit higher separation efficiencies than CZE-MS but may often also reveal better selectivity in particular for structurally closely related analytes like different charge variants as shown for hemoglobin [10]. The CIEF separation delivers information on the p*I* value which can be regarded as a valuable additional identification feature that is not available in CZE separations. The CZE-ESI-MS interface is well established. Ionization suppression effects are usually less pronounced in CZE-MS with MS compatible buffers. In contrast, CIEF suffers from ionization suppression by ampholytes, acids, and bases present in catholytes and anolytes, mentioned already above. In conclusion, the benefits of a higher separation efficiency due to the focusing effect, the more information-rich MS detector, and information about p*I* of analytes should make it worthwhile investing more efforts to overcome the technical difficulties of CIEF-MS. Supporting our attempts to develop the field further we herein, summarize developments on different approaches to couple IEF with MS. One common offline approach is the combination of strip-based IPG-separation with LC-MS. This is a similar approach as the standard proteomics workflow combining 2D GE, tryptic digestions, and LC-MS/MS and is out of scope of this paper. In this review, online and offline couplings are briefly discussed and the coupled systems are distinguished by the interface between the IEF and the MS.

2 Technical strategies for CIEF-MS

2.1 CIEF offline coupled to MALDI-MS

A general challenge by connecting a CIEF separation to MALDI-MS is the transfer of analytes to the MALDI plate without losing the high separation efficiency; the low peak volumes of narrow zones require addition of sheath liquid for efficient sample/droplet deposition on the MALDI plate which might cause diffusion effects, mixing effects and inappropriate flushing of the interface that must be avoided [11]. Most importantly, however, separation resolution in CIEF-MALDI depends on the spotting frequency which has to be carefully adjusted. Furthermore, the quality of MS spectra might be compromised by sample/matrix preparations with the catholyte-sheath solutions and residuals of the ampholytes present as compared to standard MALDI matrix preparations. While it is known that MALDI is less sensitive for additives in the sample such as salts, it was shown that MS signal intensities decreased significantly by additives like ampholytes (signal decrease of 50% by use of 1% ampholyte), hydroxy ethyl cellulose (0.3% of hydroxy ethyl cellulose decreases the MS signal by about 60%) and Tween-20 (0.1 % Tween reduces the signal by about 50%) that are commonly present in deposited sample fractions from CIEF experiments [12]. On the other hand, it might be argued that the co-crystallization process in MALDI is a purification process itself which might help avoiding unfavorable effects of ampholytes on ionization yield. However, this has not been sufficiently documented in the reported offline CIEF-MALDI-MS studies.

The first interface for offline coupling of CIEF to MALDI-TOF-MS was reported by Foret et al. [13]. Acetic acid (pH 2.9) was used on the anolytic side and ammonium hydroxide

Table 1. CIEF followed by MALDI-MS approaches

Anolyte and catholyte solution	Interface	MS (MALDI matrix)	Focusing	Mobilization	Ampholytes; p*I* range	Application	Additional information	Ref.
Acetic acid at pH 2.9 and NH_4OH at pH 10.9	Sheath flow tee interface (Fig. 1a)	MALDI-TOF-MS (Matrix: Sinapinic acid in TFA:CAN (60:80)), catholyte solution	10 kV for 2 min followed by 30 kV for 13 min	Raising inlet vial up for 5 (22 nL/min) or 8 cm (35 nL/min) + high voltage	1 % (1:1:1 v/v/v) mixture of Pharmalyte (Sigma, St. Louise, MO, USA), Ampholyte (Sigma) and Servalyte (Serva, Hauppauge, NY, USA); p*I* range: 5.1–7.2	β-lactoglobulin A, myoglobin, carbonic anhydrase I and II and hemoglobin variants	Preparative collection of analyte bands in solution or salt-free solids.	Foret et al., 1995 [13]
100 mM H_3PO_4 and 30 mM NH_4OH	Sheath flow tee interface (Fig. 1a)	MALDI-TOF-MS (Matrix: Sinapinic acid in TFA:CAN (60:80)), catholyte solution	Const. 30 μA for 10–20 min with increasing voltage	By pressure with a syringe pump at a constant flow velocity	2 % Ampholine (Sigma-Aldirch, St. Louis, MO, USA)	β-lactoglobulin A/B, myoglobin, carbonic anhydrase II, ovalbumine	Preparative collection of analyte bands.	Minarik et al., 2000 [14]
15 mM H_3PO_4 and 50 mM NH_4OH	Sheath flow tee interface (Fig. 1b)	MALDI-TOF-MS (Matrix: 10 mg/mL THAP and 10 mg/mL diamonium hydrogen citrate in methanol/H_2O 5/1 (dried droplet) or 2/1 (thin layer), catholyte solution	30 kV for 12.5–20 min	20 mbar pressure + 30 kV	2.5 % (w/v) Servalyt (Serva, Heidelberg, Germany); p*I* range: 3–10	Tryptic digest of glyco- and phosphopro-teins (e.g. ovalbumin from chicken egg and plasma derived human antihrombin)	In PVA coated and fused silica capillaries. Direct deposition of analyte drops from the sheath flow interface to the MALDI plate	Lechner et al., 2008 [11]
10 mM H_3PO_4 + HEC (0.3 %) and 200 mM NH_4OH + 50% methanol (+ 0.01 % Tween-20 for mobilization)	Sheath flow tee interface (Fig. 1b)	MALDI-TOF-MS (5 mg/mL sinapic acid in 80% v/v CAN with 0.1% v/v TFA), catholyte solution	20 kV for 15–90 min	100 mbar + 20 kV	1 % (w/v) (Pharmalyte Amersham Bioscience, Piscataway, NJ, USA); p*I* range: 3–10	Different proteins (RNase XII A, Myo, CAII, LGB, LGA, Lac, CCK)	Direct deposition of analyte drops from the sheath flow interface to the MALDI plate. Only commercial parts were used.	Silvertand et al., 2009 [12]
1% acetic acid and 1% NaOH	Membrane separated electrodes (Fig. 1c)	MALDI-TOF-MS (Matrix: Parafilm M precoated MALDI spots and DHB 120 mg/mL)	20 kV for 10–12 min	By pressure air	0.5 % Pharmalyte (Sigma Aldrich, St. Louis, MO, USA); p*I* range 3–10	BSA tryptic peptides and neuropeptides from *C. borealis* and *C. sapidus*.	Direct deposition of analyte drops without sheath liquid.	Zhang et al., 2011 [15]

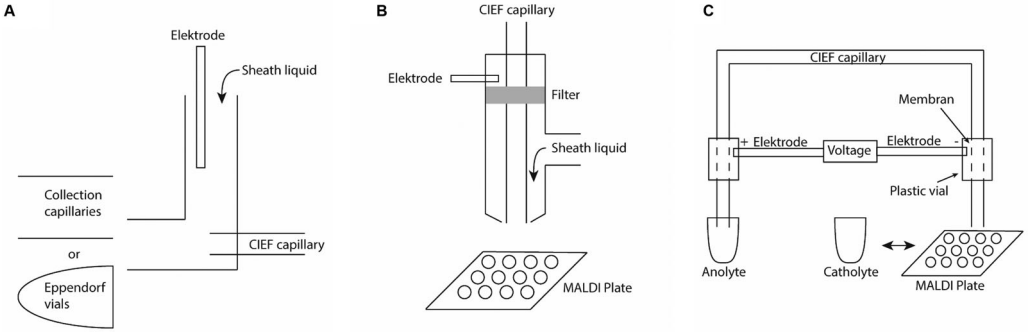

Figure 1. The three applied interfaces for coupling CIEF to MADLI-MS. (A) With preparative collection in capillaries and Eppendorf vials [13, 14], (B) by a sheath–liquid interface [11, 12], and (C) with membrane-separated electrode, anolyte, and catholyte [15].

(pH 10.9) admixed via a tee connection (sheath–liquid interface) on the catholytic side. A schematic drawing of the used tee connection containing the CIEF capillary, the electrode, and a sheath liquid connection is shown in Fig. 1a. The CIEF capillary was placed in-line to the collection capillaries. The electrode and the sheath liquid connection were placed in a 90-degree angle to the CIEF capillary. The electrode on the inlet side was separated by a membrane to reduce interference by produced air bubbles. The used focusing and mobilization conditions are described in detail in Table 1. Foret et al. were using a mixture of three commercially available ampholyte solutions to create a uniform pH gradient. For minimizing band broadening, high voltage was applied during the mobilization. The analytes were detected by on-column fiberoptic UV detection shortly before entering the sheath–liquid interface. The sheath–liquid interface was placed 200 μm in front of a rotor which possessed 60 collection capillaries with 20 μL each. The proteins were stored as solutions in the collection capillaries or as salt-free solids after the evaporation of the ammonium hydroxide solution. If stored as solutions, 2 μL of the collected proteins were deposited on the MALDI plate and allowed to evaporate. The solid fractions were dissolved in MALDI matrix. Both types of samples were analyzed by MALDI-TOF-MS successfully.

In a later work, the same group improved the concept by use of two UV detection systems close to the sheath flow tee interface [14]. With this improvement, they were able to determine the zone velocity in the capillary during the CIEF experiment and close to the sheath flow collection interface. This way, they achieved a more accurate isolation of the analyte zones. Moreover, they used 100 μm id capillaries, compared to the most commonly used 50 or 75 μm id capillaries, to increase the amount of sample which can be injected. It was demonstrated that smaller id of the capillaries leads to better resolutions while 100 μm id is the best compromise between resolution and injectable sample amount. Low conductivity buffer and a slowly increasing focusing voltage were used to reduce the Joule heating through the wider capillary id. Chemical mobilization was discarded due to the nonlinear profile of elution time versus p*I* and the higher current during the mobilization. The analyte bands were collected through the tee interface in Eppendorf vials.

Lechner et al. were using a system in which the droplets from the CIEF capillary were directly deposited on the MALDI plate by a homemade sheath liquid flow plastic tip [11]. The principal structure of this sheath liquid flow tip is displayed in Fig. 1b. The working conditions for focusing and mobilization are displayed in Table 1. The sheath flow tip was located inside a vial, containing 50 mM NH_4OH during the focusing and was repositioned during the mobilization briefly over the MALDI plate. Mobilization was performed by pressure-assisted EOF in fused silica capillaries and only with pressure in PVA-coated capillaries. During the mobilization, the sheath flow, which served as the current bridge and allowed the direct deposition of the droplets on the MALDI plate, was containing 50 mM NH_4OH. They used a droplet of 450 nL sheath liquid/analyte per spot. Five hundred nanoliters of the MALDI matrix was prespotted. The analytes were detected shortly before the end of the capillary by an on-capillary UV detector at 440 nm.

Silvertand et al. were also coupling CIEF to MALDI-MS by a direct drop deposition similar to the instrument from Lechner et al. The experimental conditions can be found in Table 1. The sheath liquid (200 mM ammonium hydroxide in 50% MeOH and 0.01% Tween-20) was delivered via a syringe coupled via tubing in 90° to the CIEF capillary and the spotting tip. The sheath liquid flow was set to 500 nL per spot. A spotting time of 5 s which leads to 500 nL per spot was investigated as the best compromise between amount of sample and effect of additives. The effect of additives were bigger than the effect of the protein concentration itself, therefore shorter spotting might lead to better signal intensities but were not possible with this instrument.

Another possibility for spotting analyte bands on a MALDI target is to separate the electrode via a membrane shortly before the end of the capillary. Zhang et al. were demonstrating this idea with a 75 μm id capillary which contains two plastic vials, each positioned 3 cm before the end of the capillary, connected via membrane to the capillary, containing the electrode and filled with 1% acetic acid [15]. A scheme of this instrumental set up can be seen in Fig. 1c. The used anolyte and catholyte solution as well as the measuring conditions are listed in Table 1. Zhang et al were placing the end of the capillary briefly over the MALDI plate during

the mobilization step. The MALDI plate was precoated with Parafilm M and prespotted with MALDI matrix. With this new instrument design, they achieved better performance in terms of separation efficiency, reduced sample loss, and improved reproducibility, because there were no air bubbles, produced by the electrodes, entering the capillary, and the sample loss due to band shifting was prevented. Furthermore, they achieved a spotting on the MALDI plate without the dilution effect of a sheath liquid flow.

CIEF followed by MALDI-MS was applied offline for standard proteins and hemoglobin variants [13, 14]. CIEF-MALDI-MS was used for the analysis of different standard proteins [12], neuropeptides [15], tryptic digests of different proteins [11], and analysis of nondegraded GlucaGen [12].

In conclusion, first offline coupling of CIEF to MALDI MS was done by Foret et al. and improved by Minarik et al. Both showed that it is possible to collect preparatively the isoelectrically focused zones. In addition to the MALDI MS measurements, this opened the possibility for Edman sequencing or peptide mapping of the stored fractions. However, this approach had to deal with long analysis time and laborious experimental lab work. An important further step was the development of an automated spotting of the separated bands on the MALDI plate which reduced the analysis time and the lab work dramatically. This way, it is possible to conserve the high-resolution power of the CIEF separation better than the offline approaches which were done before. This allowed the analyses of more complex samples. Zhou et al. received higher analyte concentration on the MALDI spot by sheath flow-free coupling of CIEF to MALDI-MS and were able to improve the separation efficiency and reproducibility by the membrane-based separation of electrode and sample. In general, using a MALDI-MS system gives the possibility to conserve the separation for later remeasurements but all of the concepts above had to deal with the limited resolution and sensitivity for larger analytes, interference from ampholytes, and limited separation efficiency under more MS friendly conditions.

2.2 CIEF-MS with catholyte/spray solution exchange after focusing for mobilization of analytes into MS

Tang et al. demonstrated for the first time the online coupling of CIEF to an ESI–MS interface by placing the catholyte vial inside the electrospray housing during the focusing [9]. This concept is schematically outlined in Fig. 2a. For the electrophoretic mobilization of the zones toward the detector, the outlet tip of the capillary was fixed 0.5 mm outside the electrospray needle within a sheath liquid sprayer (see also Fig. 2d). Using such an instrumental setup, the influence of the ampholyte concentration on the CIEF separation and the ionization process was investigated. All measuring parameters are summarized in Table 2. Protein zones were monitored by UV detection. Minimizing the movement of the ionic boundary, which leads to an increase of the separation

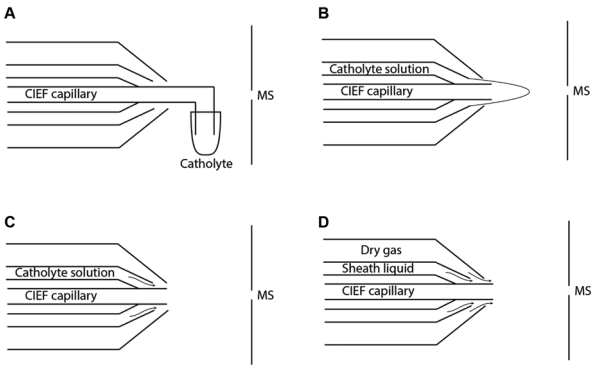

Figure 2. CIEF–ESI interface for the direct coupling of CIEF to MS. (A) With the catholytic vial placed inside the ESI housing during the focusing step [9,10,16,18–21], [37–39], [39–42], [44]. (B) Using of a build droplet of catholyte solution which was delivered through the sheath–liquid channel for the focusing process [17]. (C) ESI interface during the focusing process when the catholyte solution is delivered as through the sheath–liquid channel and the capillary is placed in the MS-ready position (0.5–1 mm outside the ESI needle) [22–24]. (D) State of ESI interface for mobilization and MS detection which was applied after the focusing process in state (A), (B), or (C).

efficiency, was achieved by employing gravity and catholytic mobilization of the focused analytes. In order to elucidate the influence of the ampholyte concentration, it was varied in the range between 5 and 0.1%. A reduction of the ampholyte concentration resulted in a decrease of resolution (–22% from 5 to 0.5% ampholyte concentration) and a reduction of the UV absorbance intensity (–34 from 5 to 0.5% ampholyte concentration). On contrary, a high ampholyte concentration led to ion-pair formation of the ampholyte with the proteins. The combination of ion-pair formation and subsequent desolvation and dissociation processes led to charge neutralization and a shift in the charge distribution of the protein mass spectra. The best ampholyte concentration was determined to be 0.5%. It was demonstrated that the principle of this direct CIEF-ESI hyphenation works and can achieve LODs which are two orders of magnitude smaller than with CZE for standard proteins. However, because of the exchange of the outlet vial by the ESI sprayer, this approach is not automated and the influence of ampholytes still exists. In order to speed up the protein mobilization and simultaneously minimizing the moving ionic boundary inside the CIEF capillary, Yang et al. supported the electrophoretic mobilization by gravity (lifting up the inlet vial by 8 cm) [16].

In order to allow analysis of salt-rich biological samples (like digests) by CIEF-MS, desalting of the samples prior to the CIEF analysis is necessary. Clarke et el. were the first to introduce an online desalting inside the CIEF capillary without any additional equipment [17]. The desalting was done after filling the capillary with the sample and ampholyte solution by applying a voltage gradient from 0–10 kV over 6 min. During this time, a sharp current drop was observed which marked the point at which the salt plug has left the capillary. The analysis was carried out in a 50 μm PVA-coated capillary

Table 2. CIEF-MS with catholyte/ESI exchange after focusing for mobilization of analytes into MS and direct CIEF-MS approaches

Anolyte and catholyte solution	Interface	MS; sheath liquid (flow)	Focusing	Mobilization	Ampholytes; p*I* range	Application	Additional information	Ref.
20 mM H_3PO_4 and 20 mM NaOH	ESI housing (Fig. 2a–d)	ESI-QqQ-MS; 50% methanol, 49% H_2O, and 1% acetic acid (3 μL/min)	10 kV for 15 min	10 kV or 500 V/cm	0.5 % Carrier ampholytes, Pharmalyte (Pharmavia, Uppsala, Sweden); p*I* range: 3–10	Cytochrome c (horse heart), myoglobin (horse heart), and carbonic anhydrase II (bovine erythrocyte)	Effect of pharmalyte concentration on protein separation in CIEF	Tang et al., 1995 [9]
20 mM H_3PO_4 and 20 mM NaOH	ESI housing (Fig. 2a and d)	ESI-QqQ-MS; 50% methanol, 49% H_2O and 1% acetic acid (3 μL/min)	10 kV for 15 min	10 kV or 500 V/cm	0.5 % Carrier ampholytes, Pharmalyte (Pharmacia, Uppsala, Sweden); p*I* range: 3–10	Cytochrome c (horse heart), ribonuclease A (bovine pancreas), myoglobin (horse heart), carbonic anhydrase I (bovin erythrocyte) and β-lactoglobulin A (bovine milk)	CIEF	Tang et al., 1996 [10]
20 mM H_3PO_4 and 20 mM NaOH	ESI housing (Fig. 2a and d)	ESI-QqQ-MS; 50% methanol, 49% H_2O, and 1% acetic acid (3 μL/min)	10 kV for 15 min	10 kV or 500 V/cm	0.5 % Carrier ampholytes, Pharmalyte (Pharmacia, Uppsala, Sweden); p*I* range: 3–10	Hemoglobin variants A, C, S, and F		Tang et al., 1996 [38]
20 mM H_3PO_4 and 20 mM NaOH	ESI housing (Fig. 2a and d)	ESI-QqQ-MS; 50% methanol, 49% H_2O, and 1% acetic acid (3 μL/min)	10 kV for 15 min	10 kV or 500 V/cm + raise up 8 cm of inlet vial	0.5% Carrier ampholytes, Pharmalyte (Pharmacia, Uppsala, Sweden); p*I* range: 5–8	Bovine serum and apotransferrin glycoforms	Gravity-induced hydrodynamic flow. Comparison CIEF vs CZE	Yang et al., 1996 [16]
20 mM H_3PO_4 and 20 mM NaOH	ESI housing (Fig. 2a and d)	ESI-QqQ-MS; 50% methanol, 49% H_2O, and 1% acetic acid (3 μL/min)	10 kV for 15 min	10 kV or 500 V/cm + raise up of inlet vial	0.5% Carrier ampholytes, Pharmalyte (Pharmacia, Uppsala, Sweden); p*I* range: 5–8	Recombinant *E. coli* proteins	Comparison CIEF vs 2D Gel IEF	Tang et al., 1997 [37]
50% methanol, 49% H_2O, 1% acetic acid (1%) and 50% methanol, 49% H_2O, 1% NH_4OH	ESI housing, hanging droplet (Fig. 2b and d)	ESI-Sector field-MS; Anolyte solution (3 μL/min)	20 kV for 7 min	20 kV	0.5 % Servalytes (Sigma Chemical Co., St. Louis, MO, USA); p*I* range: 3–10	Cytochrome c (horse heart), myoglobin (equine skeletal muscle), carbonic anhydrase I (bovine erythrocyte) and trypsin inhibitor (soybean) and physiologically derived fluids	Online desalting by a voltage gradient after filling the capillary with sample. Hanging droplet.	Clarke et al, 1997 [17]

Table 2. Continued

Anolyte and catholyte solution	Interface	MS; sheath liquid (flow)	Focusing	Mobilization	Ampholytes; p*I* range	Application	Additional information	Ref.
20 mM H_3PO_4 and 20 mM NaOH	ESI housing (Fig. 2a and d)	ESI-QqQ-MS; 50% acetone, 49% H_2O, and 1% acetic acid (3 μL/min)	15 kV	20 kV + raise up of inlet vial	0.5% Pharmalyte; p*I* range: 4–6.5	Protein phosphorylation		Wei et al., 1998 [39]
20 mM H_3PO_4 and 20 mM NaOH	ESI housing (Fig. 2a and d)	ESI-QqQ-MS; 50% acetone, 49% H_2O and 1% acetic acid (3 μL/min)	10 kV	15 kV + raise up 10 cm of inlet vial	0.5% carrier ampholytes, Pharmalyte (Pharmacia, Uppsala, Sweden); p*I* range: 3–10	Monitoring protein refolding induced by disulfide formation		Jensen et al., 1998 [40]
0.5% acetic acid and 0.5% NH_4OH	ESI housing (Fig. 2a and d)	ESI-IT-MS; 75% methanol, 25% H_2O, and 0.25% acetic acid (1.5–2.5 μL/min)	25–30 kV for 5–7 min	25–30 kV + 0.1 psi inlet pressure	1–2% Ampholine (Pharmacia Biotech, Piscataway, NJ, USA); p*I* range: 3.5–10	High affinity ligands to the Src SH2 domain		Lyubarskay et al., 1998 [41]
20 mM H_3PO_4 and 20 mM NaOH	ESI housing (Fig. 2a and d)	ESI-FTICR-MS; 50% methanol, 49% H_2O, and 1% acetic acid (3 μL/min)	15 kV for 10 min	19 kV + raise up 8 cm of inlet vial	0.5% Carrier ampholytes, Pharmalyte (c, Uppsala, Sweden); p*I* range: 3–10 and 5–8	Cytochrome c (horse heart), myoglobin (horse heart), carbonic anhydrase I and II (bovine erythrocyte), human hemoglobin variants A, C, S, F, and *E. coli* lysate proteins	First use of FTICR-MS	Yang et al., 1998 [18]
20 mM H_3PO_4 and 20 mM NaOH	ESI housing (Fig. 2a and d)	ESI-FTICR-MS; 50% methanol, 49% H_2O, and 1% acetic acid (2 μL/min)	8 kV for 10 min	8 kV + raise up 5 cm of inlet vial	0.5 % Pharmalyte (Amersham Pharmacia Biotech, Piscataway, NJ, USA); p*I* range: 3–10	Proteins from *E. coli*	Comparison of CIEF versus 2D PAGE	Jensen et al., 1999 [21]
20 mM H_3PO_4 and 20 mM NaOH	ESI housing (Fig. 2a and d)	ESI-FTICR-MS; 50% methanol, 49% H_2O, and 1% acetic acid (2 μL/min)	25–30 kV	25–30 kV	1 % Pharmalyte (Amersham Pharmacia Biotech, Piscataway, NJ, USA); p*I* range: 3–10	Intact noncovalent protein–protein complexes		Martinovic et al., 2000 [42]
20 mM H_3PO_4 and 20 mM NaOH	ESI housing (Fig. 2a and d)	ESI-FTICR-MS; 50% methanol, 49% H_2O, and 1% acetic acid (2 μL/min)	8–13 kV for 10 min	8–13 kV + raise up 5 cm of inlet vial	0.5 % Pharmalyte (Amersham Pharmacia Biotech, Piscataway, NJ, USA); p*I* range: 3–10	Cell lysate from *E. coli* and *D. radiodurans*	Analytes with p*I* < 0.004–0.005 were separated	Jensen et al.,2000 [20]
20 mM H_3PO_4 and 20 mM NaOH	ESI housing (Fig. 2a and d)	ESI-FTICR-MS; 50% methanol, 49% H_2O, and 1% acetic acid (2 μL/min)	8–13 kV for 10 min	8–13 kV + raise up 8 cm of inlet vial	1 % Pharmalyte (Pharmacia Diagnostics (Upsala Sweden)); p*I* range: 3—10	High-throughput proteomics for several microorganisms		Smith et al., 2001 [19]

Table 2. Continued

Anolyte and catholyte solution	Interface	MS; sheath liquid (flow)	Focusing	Mobilization	Ampholytes; p*I* range	Application	Additional information	Ref.
50 mM ammonium acetate (pH 9.3) and 50% methanol, 49% H_2O, 1% acetic acid	Sheath liquid interface during focusing (Fig. 2c and d)	ESI-FTICR-MS; Anolyte solution (2 μL/min)	25-30 kV	25-30 kV + different pressure for stepwise mobilization	Carrier ampholytes, Pharmalyte (Pharmavia, Uppsla, Sweden); p*I* range: 3–10	Bovin cytochrome C, horse myoglobin, bovin carbonic anhydrase II, and *E. coli* lysate	First direct coupling without repositioning of capillary and turning off the voltage. Fused silica capillary.	Zhang et al., 2000 [22]
1% acetic acid and 0.28% NH_4OH	Sheath liquid interface during focusing (Fig. 2c and d)	ESI-TOF-MS; 50% methanol, 49% H_2O, and 1% acetic acid (2 μL/min)	20 kV for 10 min	20 kV	0.5–4% Pharmalyte; p*I* range 3–10	Quantitative analysis of tetrasialo-transferrin	Change of catholyte to sheath liquid between focusing and mobilization	Kuroda et al., 2005 [24]
50 mM or 125 mM formic acid in 30% glycerol/H_2O and 100 mM NH_4OH in 30% glycerol/H_2O	Flow-through microvial interface (Fig. 2c and d)	ESI-IT or Q-TOF-MS; Methanol, H_2O and formic acid in different mixing ratios (0.3 μL/min)	30 kV	30 kV	1–2% Ampholytes	RNase A, Myoglobin, carbonic anhydrase II, and β-lactoglobulin, CCK peptide	Direct coupling, fused silica capillary, "sandwich injection"	Zhong, 2011

which was fixed at the outlet end inside the ESI sprayer. Analyte, catholyte solution, and separation conditions are listed in Table 2.

The catholytic solution was delivered at 1 μL/min through the sheath liquid channel in a way that a "hanging" droplet was produced at the tip of the capillary which is also displayed in Fig. 2b. After the focusing of the peaks, the capillary was fixed 0.5 mm outside the ESI sprayer tip and the sheath liquid was changed to the anolyte solution which is assisting the ESI process (see also Fig. 2d).

Up to this point all described CIEF-ESI-MS couplings were accomplished with a triple quadrupole-MS. To achieve a higher mass resolution, Yang et al. [18] were the first hyphenating CIEF to a Fourier transform ion cyclotron resonance (FTICR)-MS employing the direct CIEF-ESI-MS presented by Tang et al. [9]. Favorably, also lower LOQ values were obtained. All experimental parameters adopted by Yang et al. and all other applications which utilized the same hyphenation technique as proposed by Tang et al. are summarized in Table 2.

Various applications of the CIEF-ESI-MS coupling technique described by Tang et al. with triple quadrupole, IT, or FTICR-MS, ranging from standard proteins and cell lysates to characterization of noncovalent protein complexes from mixtures were reported. Of particular interest are the studies by Smith et al. and Jensen et al. [19–21]. They utilized a CIEF-FTICR-MS system and were able to detect in a single run, around 210 discrete peaks or 900 unique putative protein masses with p*I* differences down to 0.004–0.005 from a cell lysate. A 2D virtual gel display of a tryptic digest of soluble *D. radiodurans* and *Escherichia coli* is shown in Fig. 3. The spectrum from phosphoglycerate kinase obtained from CIEF-FTICR-MS analysis can be seen in Fig. 4.

In conclusion, the technique placing the catholyte vial inside the ESI housing for focusing, and switching to ESI sprayer and sheath liquid for mobilization as well as detection, developed by Tang et al., 1995 is an interesting approach for fast and reproducible CIEF-ESI-MS analysis with impressive results. It is by far the most frequently used technique. It was further improved by gravity-assisted mobilization and the use of high-resolution MS detectors. In addition, many influential factors like ampholyte concentration and moving ionic boundary were investigated deeply and the results were used for further improvements. However, all applications with this technique had one capillary replacing step and a voltage interruption between focusing and mobilization. This may result in a loss of separation efficiency and hinders automation. A first step toward automation was done by delivering the catholytic solution through the sheath–liquid interface which then produces a "hanging" droplet at the capillary tip. Still, the change of the sheath liquid, a repositioning of the capillary and an interruption of the voltage was necessary.

2.3 Direct CIEF-MS

To avoid a loss of separation efficiency, Zhang et al. demonstrated the possibility of placing the CIEF capillary inside the

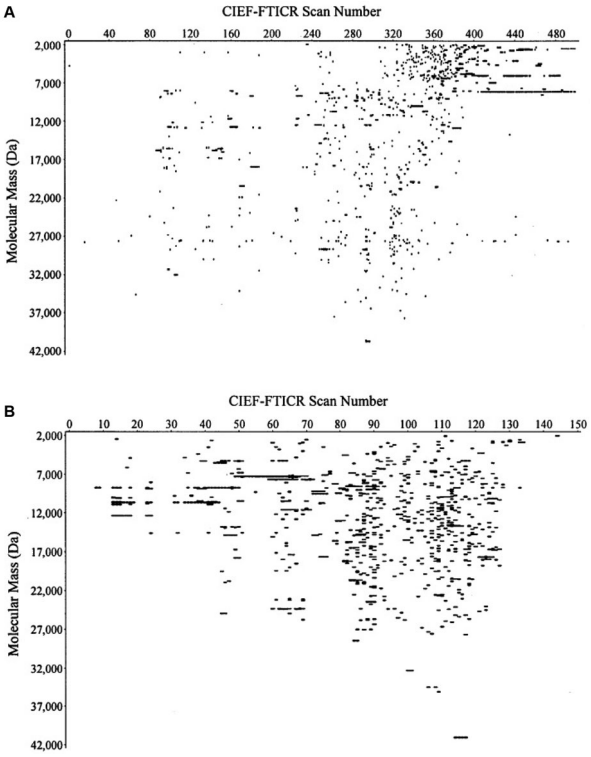

Figure 3. 2D display of the CIEF-FTICR analysis of lysates from (A) *D. radiodurans* (B) *Escherichia coli*. Reprinted with permission from [20]. Copyright © 2015 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

ESI sprayer in the MS-compatible arrangement during the focusing process [22]. In this case, the sheath liquid (0.2 M acetic acid in 50% (v/v) methanol) acts as the catholyte during the focusing step (Fig. 2c) and as the sheath liquid during the mobilization and MS detection step (Fig. 2d). Utilizing this configuration it was possible to mobilize the analytes without interruption of the voltage. Zhang et al. were also using a stepwise mobilization by the application of a reversed EOF generated by the carrier ampholytes in uncoated capillaries and different inlet pressure values. They showed that the EOF can be used to the benefit of the separation and is not necessarily a problematic attendant phenomenon. Catholytic and analytic solutions as well as focusing and mobilization parameters are summarized in Table 2. Zhong et al. focused on the development of a hyphenation strategy which keeps the present advantage without the need for special instrumentation and provides the possibility for automation [23]. The experimental conditions from Zhong et al. are summarized in Table 2. A flow-through microvial, which is shown schematically in Fig. 2c, between the end of the capillary and the interior of the needle tip was used. Ensuring a constant electric contact, a fast switch between catholytic solution during focusing and acidic solution for the electrophoretic mobilization could be reached. "Sandwich" injection (A), focusing (B), and mobilization (C) toward the MS with a modifier, which acts as the sheath liquid for the ESI process, is displayed in

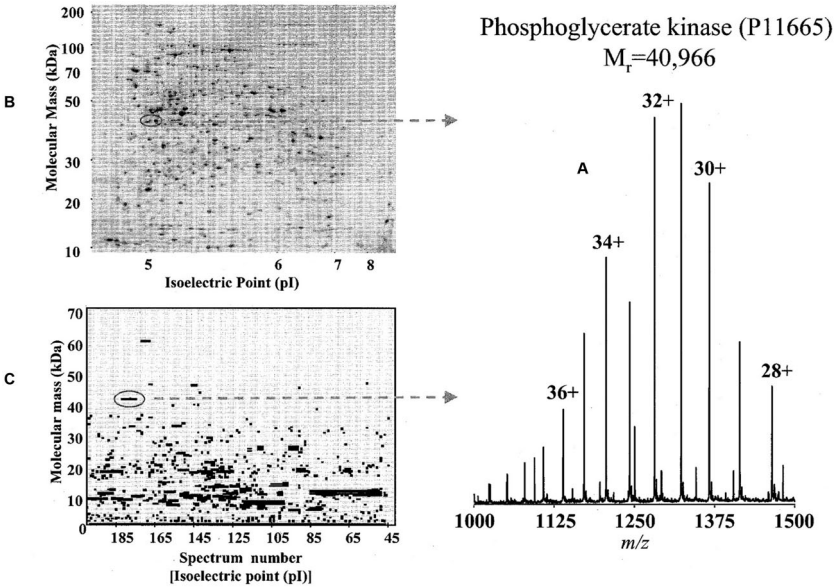

Figure 4. (A) Positive ion mode mass spectrum for phosphoglycerate kinase obtained from CIEF-FTICR analysis of the lysate from *Escherichia coli* grown in isotopically depleted media. (B) The 2D PAGE for *E. coli* reproduced from SWISS-2D PAGE, Swiss Institute of Bioformatics (http://www.expasy.ch/ch2s/). (C) 2D display of CIEF-FTICR results for a lysate of *E. coli* grown in isotopically depleted media. Reprinted with permission from [21]. Copyright 2015 American Chemical Society.

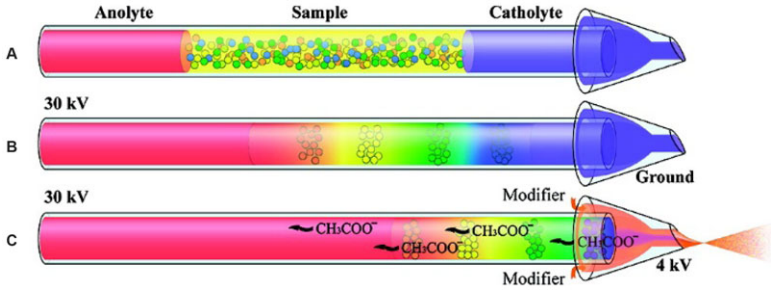

Figure 5. Scheme of the flow-through microvial during the (A) injection (B) focusing, and (C) voltage mobilization. Reprinted with permission from [23]. Copyright 2015 American Chemical Society.

Fig. 5A–C. The "sandwich" injection (Fig. 5A) means that catholyte, sample, and anolyte were injected subsequently in individual plugs. The short sample zone in this "sandwich" approach ensured that the focusing was complete before the EOF was pushing the ampholyte zones out of the capillary. Reasonable separation efficiency was achieved with CIEF in bare-fused silica and neutral-coated capillaries, respectively. The EOF in the bare-fused silica capillaries was part of the mobilization strategy increasing together with glycerol as an anticonvective agent, the resolution, and relative ESI-MS sensitivity compared to the classical pressure mobilization.

The direct CIEF-ESI-MS coupling was used for the analysis of cell lysates with the detection of more than 100 putative protein peaks with masses from 6 to 60 kDa [22]. A second application was the quantitative determination of tetrasialo-transferrin and a quantification of Angiotensin II with an LOD of 0.22 μM, which was ten times lower than with UV detection [24].

In conclusion, it is possible to establish an online hyphenation of CIEF and ESI-MS by using the sheath liquid as the catholytic solution during the focusing step [25] [23]. Using a flow-through microvial, glycerol as a modifier and an EOF during the mobilization step, the resolution and the sensitivity could be improved. In this way, Zhong et al. provided a fully automated process due to the application of commercial CE and MS instruments. However, a separation of ampholytes and analytes was not achieved and therefore an interference of the ESI process by the ampholyte could not be fully prevented.

2.4 CIEF-MS with partial ampholyte filling technique

One way to improve signal intensities in online CIEF-ESI-MS coupling is the use of discontinuous ampholyte zones. Thereby, it can be prevented that ampholytes and analytes arrive at the same time at the ESI interface. Páger et al. demonstrated a successful CIEF-MS hyphenation using this approach by separate injection of the sample and the ampholyte [26]. All general parameters are summarized in Table 3. In this work, two different injection protocols were tested for the separation of amphoteric dyes: In the first injection mode, a 40-s injection of ampholytes was followed by a 6-s injection of the sample and finally a 40-s injection of the ampholytes. In the second injection mode, they only injected the sample for 6 s followed by a 40-s injection of the ampholytes. They discovered that one ampholyte zone after the sample zone is enough for a separation of the analyte components. With this setup, the analytes can reach the ESI source in a zone which is devoid of disturbing ampholytes.

Table 3. CIEF-MS with partial filling, point focusing, immobilized pH gradient, or a micro dialysis unit approaches

CIEF-MS with partial filling, point focusing, immobilized pH gradient, or a micro dialysis unit								
Anolyte and catholyte solution	Interface	MS; Sheath liquid (flow)	Focusing	Mobilization	Ampholytes; p*I* range	Application	Additional information	Ref.
50 mM formic acid and 50–100 mM ammonium hydroxide	Multiple junction capillary	ESI-IT; methanol and:H_2O 1:1 (10 μL/min)	200–300 V/cm	50 mbar + high voltage	Ampholine (LKB, Bromma, Sweden); p*I* range: 4–6, 7—9, and 3.5-10	Amphoteric dyes	Only ampholyte bands for and after analytic bands	Páger et al., 2011 [26]
		ESI-PenTOF and Orbitrap XL MS; acetonitrile, water, and formic acid (6:3:1)	400–8 kV	1–3 μL/min of different buffer solutions	Well-defined buffer solutions (ammonium formiate + formic acid or ammonium hydroxide); pH range: 2.9–8.5	B-lactoglobulin A, hemoglobin A, myoglobin, α-chymotrypsinogen A, ribonuclease A, cytochrome c, and lysozyme.	Multiple-junction capillary (Isoelectric point focusing)	Chingin et al., 2012 [27]
1% formic acid and 50% 2-propanol, 49% water and 1% formic acid	Homemade sheath liquid interface	ESI-IT MS; catholyte solution (250 μL/h)	350–400 V/cm	By pressure with 50% 10 mmol/L ammonium formate/50% ACN	Immobilized ampholines	Low molecular weight acids	Monolytic immobilized pH gradient	Wang et al., 2011 [28]
2% acetic acid as weak acid and weak base	Microdialysis device	ESI-Q-MS; methanol and 5% acetic acid (80:20)	30 kV	30 kV + 25 mbar	1 or 2.5% Pharmalyte; p*I* range: 5–8	myoglobin, β-lactoglobulin A, and carbonic anhydrase I	Dialysis interface	Lamoree et al., 1997 [29]
10 % acetic acid and 0.3% ammonium hydroxide	Microdialysis device	ESI-QqQ-MS; 50% methanol, 49% H_2O, and 1% acetic acid (0.3 μL/min)	15 kV	13 kV and 0.2 psi nitrogen pressure at inlet	Carrier ampholytes, Pharmalyte (Pharmacia, Uppsala, Sweden); p*I* range: 3–10	Cytochrome c (horse heart), myoglobin (horse heart) and carbonic anhydrase II (bovine erythrocyte)	Dialysis interface	Yang et al., 1998 [30]

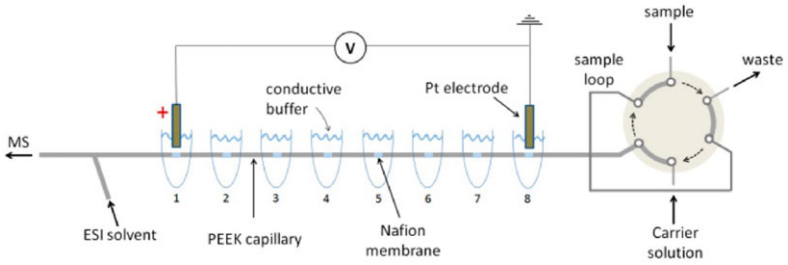

Figure 6. Schematic layout of the online multiple junction – CIEF setup: the six-port injector is shown in the sample-loop loading position. Reprinted with permission from [27]. Copyright 2015 American Chemical Society.

However, this concept was only demonstrated for amphoteric dyes and is applicable only to cases where the p*I* values of the ampholytes are just below and above of the p*I* of the analytes. Moreover, it is expected that this concept is not working for more complex samples.

2.5 Isoelectric point focusing in a multiple-junction capillary–MS

In order to overcome the general issues regarding the coupling of IEF with an MS detector, Chingin et al. developed a p*I* focusing in a multiple-junction capillary [27]. In this setup, displayed in Fig. 6, the capillary was separated in seven sections, each with a well-defined p*I* cut-off value and joint via tubular nafion membrane insertion. Each junction communicated with its own electric buffer, which enables a local buffer exchange and the use of a tailored pH profile along the entire capillary. All experimental parameters are listed in detail in Table 3. With a voltage gradient across certain segments, a selective mobilization of each fraction is possible. They designated their system as affordable, easy to fabricate, and simple in operation. The system developed by Chingin et al. represents a highly interesting approach with many different possibilities to improve the process, e.g. local buffer exchange and voltage gradient. However, this instrument set up was not applied to more complex samples so far. In addition, adsorption effects of analytes at the different membranes have to be ruled out for every analyte.

2.6 CIEF-MS with an immobilized pH gradient

Another way to avoid the entrance of ampholytes into the ESI-MS is to immobilize the ampholytes [28]. This particular IPG format was prepared by reaction of the amino groups of ampholine with the epoxy groups of glycidyl methacrylate in the presence of formamide. After adding *N,N*-methylene bisacrylamide, PEG-8000, PEG-10000, acrylamide, and azobisisobutyronitrile the solution was vortexed, degassed for a few minutes, filled into a capillary which was prior coated with 3-(trimethoxysilyl) propyl methacrylate. Afterward, the reaction mixture was allowed to polymerize to obtain the immobilized monolithic ampholyte. A 20 mmol/l glutamic acid was utilized as anolyte buffer and 20 mmol/l NaOH as catholyte buffer. The pH gradient was established with 400 V/cm for 6 min. The sample was injected and the amphoteric acids focused in an electric field. The sheath liquid of the ESI sprayer served as catholyte solution. The focused components were mobilized by pressure toward the MS detector. All additional analysis parameters are summarized in Table 3. Good efficiency was achieved by this monolithic ampholyte column to separate low molecular mass amphoteric acids. This method provides a possible solution for solving problems which arise from ampholytes in the ion source, however, was not proven for a wider range of analytes. Moreover, the approach has also some disadvantages: (i) The monolithic bed disperses the focused analyte zones in the course of the mobilization step which may lead to a significant loss in efficiencies. (ii) Interaction of analytes with the monolithic stationary phase causes a mixed chromatographic-IEF separation mode blurring the information on p*I* values provided by a plain IEF separation mechanism.

2.7 CIEF-MS with integrated microdialysis unit

A further approach to separate the disturbing components (e.g. ampholytes, strong acids, or bases) from the analytes after the IEF process is the use of a membrane or a dialysis device integrated between separation capillary and ESI source. An overview of this set-up during injection, focusing, and mobilization is shown in Fig. 7. Along this line, Lamoree et al. connected two coated capillaries via a microdialysis unit which was utilized to separate the ampholytes with a molecular mass < 650 Da from the macromolecular analytes by dialysis [29]. The analytes of interest were not able to pass through the dialysis membrane and were transported by pressure to the MS detection system. In addition, the often used phosphoric acid anolyte as well as the commonly employed sodium hydroxide catholyte were replaced by acetic acid (2%) which can function as anolyte and catholyte solution. Due to its volatility it does not exert any disturbing effects on the ESI process.

Moreover, Yang et al. investigated the performance and capability of the integrated microdialysis membrane approach in more detail [30]. They improved the concept by implementing a postseparation acidification, which enhanced the protonation and the ionization efficiency of focused proteins while maintaining the separation efficiency. However, this resulted in less sensitivity due to significant dilution.

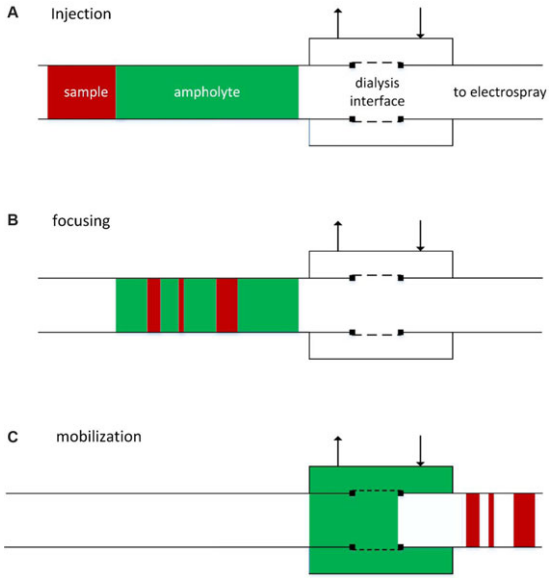

Figure 7. CIEF-MS with an integrated micro dialysis unit in the three steps of the CIEF process: (A) injection, (B) focusing, and (C) mobilization with separation of ampholytes and analytes. Adapted from [29].

An overview about the parameter, like anolyte, catholyte solution, focusing, and mobilization voltage of both CIEF-MS approaches using a dialysis interface can be found in Table 3.

In conclusion, both above-described concepts of hyphenating CIEF with ESI-MS via a dialysis membrane to separate the analytes from the disturbing ampholytes were demonstrated only for model proteins. Lamoree et al. proved that no zone broadening occurred in the dialysis interface but zone broadening was observed in the transfer capillary toward the ESI sprayer [29]. Yang et al. avoided this problem by postseparation acidification [30]. The application of a dialysis membrane is a successful way to get rid of the ampholytes, but the concept has to be proven for a larger variety of analytes with lower LODs.

2.8 CIEF-LC-MS

One way to get rid of all components interfering with analyte ionization is applying an LC system coupled between the CIEF and the ESI-MS instrument. In order to couple the CIEF online with the LC system, a valve, a membrane, or a dialysis interface is needed to connect the electrode to the CIEF separation system. Chen et al., were using a six-port-valve with a 400 nL injection loop to get on one hand an electric cycle for the CIEF focusing and on the other hand a possibility to inject the focused bands into the LC system [31]. The schematic layout of the most important parts of this instrument setup can be found in Fig. 8a. After injecting the samples by pressure into the LC system, the analytic bands were trapped in a trap column for washing away the ampholytes. All CIEF and LC conditions are summarized in Table 4.

To avoid a current cycle through the valve and injection loop, Zhou and Johnston were building up a microdialysis membrane-based cathodic cell around a PEEK tee [32]. This system is displayed in Fig. 8b. With this set up it was possible to analyze with HPLC the whole p*I* range, produced by the ampholytes, without the need of additional spacers. After focusing in the capillary, the bands were transported to two six-port valves, where the second valve contained one C18 trap column for washing away (most of) the ampholytes. Subsequently, the trapped bands were eluted to a C4 column to achieve further separation before entering the ESI-Q-TOF-MS. All measuring parameters from the CIEF and HPLC process are listed in Table 4.

In 2007, Zhou et al. advanced their system by using acetic acid, instead of nonvolatile phosphoric acid and ammonium hydroxide as anolyte and catholyte solutions, respectively. Moreover, they utilized a ten-port valve and a ten-port loop system to avoid sample loss during sampling [33]. Afterward, the samples were eluted to a C4 trap column, separated from the ampholytes, and further analyzed by a C18 column and a Q-TOF instrument as described above. All important parts of the improved concept from Zhou et al. are displayed in Fig. 8c. Zhou et al. were searching for the best combination of C4 and C18 for trap and analytical column. Moreover, they examined the influence of acetic and trifluoracetic acid. The best combination for their application and all other measuring details are described in Table 4.

Another possibility to connect the catholytic solution and electrode from the CIEF system in a way that it is possible to transport the focused bands further to an LC system was used by Wang et al. [34]. They separated the catholytic solution and the outlet electrode by a dialysis membrane from the capillary in a similar way as used by Zhang et al., 2011 for the MALDI-CIEF-MS system (described in chapter 4.1). As displayed in Fig. 8d, an injection loop and a monolithic trap column were placed after the dialysis membrane. All trapped bands were digested online with an immobilized trypsin microreactor and analyzed in parallel with two monolithic nano-RPLC systems and a linear IT mass spectrometer. They used two parallel LC systems, with two identical 3-h-long LC runs to shorten the complete analysis time (sample preparation with an overnight step followed by 30-min CIEF run and finally 3-h LC run). The used solutions for the CIEF and LC analysis are summarized in Table 4. The use of a dialysis interface provides a good solution for the problematic issue of the cathodic vial, which resides at the end of the capillary in a standalone IEF system. However, a validation of the adsorption effects on the membrane is mandatory for each analyte, respectively.

The combination of CIEF and LC-MS was applied, beside standard proteins, for different complex proteomic research samples like samples from cell lysis of *Chlorobium tepidum* which were measured by this experimental setup and compared to 2D-PAGE. The comparison is displayed as an overlapped p*I* versus MW chart from the result of

Table 4. CIEF-LC-MS approaches

IEF-LC-MS								
Anolyte and catholyte solution	Interface	MS; LC condition	Focusing	Mobilization	Ampholytes; p*I* range	Application	Additional information	Ref.
0.1 M acetic acid and 0.5% NH_4OH	6-port valve with 400 nL injection loop and trap columns (Fig. 8a)	ESI-MS/MS; Water:ACN (+ 0.02% HFBA); gradient; C18 capillary column	300 V/cm for 30 min	Hydro dynamically by acetic acid 0.1%	2% Pharmalyte (Amersham Pharmacia Biotec, Uppsala, Sweden); p*I* range: 3–10	Tryptic digest of soluble proteins from *Saccharomyces cerevisiae*		Chen et al., 2003 [31]
91 mM H_3PO_4 and 20 mM NaOH	Microdialysis membrane-based cathodic cell in a PEEK tee 3-port-valve; 6-port valve with 1 μL injection loop; C18 trap column (Fig. 8b)	ESI-QTOF-MS; Eluent A: 94.9% H_2O, 5% ACN, 0.1% acetic acid; Eluent B: 94.9% ACN, 5% H_2O, 0.1% acetic acid; Gradient; C4 column	300 V/min for 4 min	0.9 psi + 300 V/cm + raise of inlet vial	1.2 % Ampholyte (eCap cIEF, Beckman Coulter, Inc., Fullerton, CA, USA); p*I* range: 3–10	Ribonuclease A, cytochrome C, myoglobin, insulin, β-lactoglobulin, carbonic anhydrase II, bovine serum albumin, and CCK flanking peptide		Zhou et al., 2004 [32]
100 mM acetic acid and 0.5 % NH_4OH	Microdialysis membrane-based cathodic cell, 2 injection loops, 10 storage loops, C4 trap column (Fig. 8c)	ESI-QTOF-MS; Eluent A: 94.9% H_2O, 5% ACN, 0.1% TFA; Eluent B: 94.9% ACN, 5% H_2O, 0.1% TFA; Gradient; C18 widebore column	300 V/cm for 6 min	2 psi + 300 V/cm	4% Ampholyte (eCap cIEF, Beckman Coulter, Inc., Fullerton, CA, USA); p*I* range: 3–10	Intact protein profiling of chlorobium tepidum	Test of different C4 C18 combinations for trap and analytical column	Zhou et al., 2007 [33]
0.1 M acetic acid and 0.5% NH_4OH	Valve; C18-trap columns	ESI-linear IT-MS, ACN:H_2O; gradient; C18 analytical column	300 V/cm for 30 min	Hydro dynamically + 300 V/cm	2 % Ampholyte (Sigma, St. Louis, MO, USA); p*I* range: 3–10	Membrane proteins of microdissected ovarian tumor tissue	3 parallel LC systems	Wang et al., 2007 [43]
1% acetic acid and 1% NH_4OH	Membrane-based cathodic cell, injection loop, monolytic trap column (Fig. 8d)	MALDI-TOF-TOF-MS and ESI-linear IT-MS; Eluent H_2O + 0.1% formic acid; Eluent B: ACN, 0.1% formic acid; Gradient; monolytic HPLC column	300 V/cm	Raising up inlet + 300 V/cm	0.6 % Pharmalytes (Sigma, Steinheim, Germany); p*I* range: 3.5–10.0	BSA, myoglobin, β-lactoglobulin, ribonuclease A and protein extract from *E. coli*	Immobilized trypsin micro reactor between CIEF and LC-MS	Wang et al., 2011 [34]

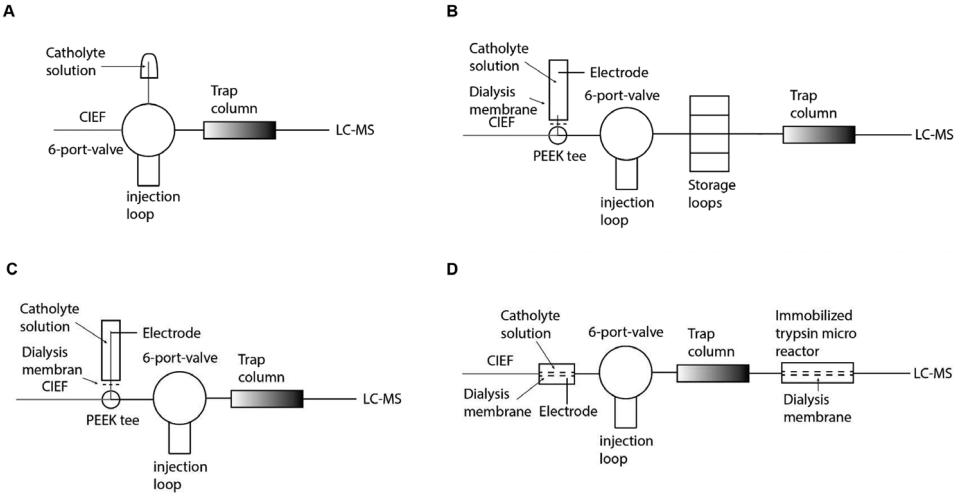

Figure 8. Different instrument setups for the online combination of CIEF with LC-MS. Focus on the connection of catholyte solution and electrode [31–34, 43]. For better overview, additional valves for the connection of pump, waste, and the storage in different loops are not shown.

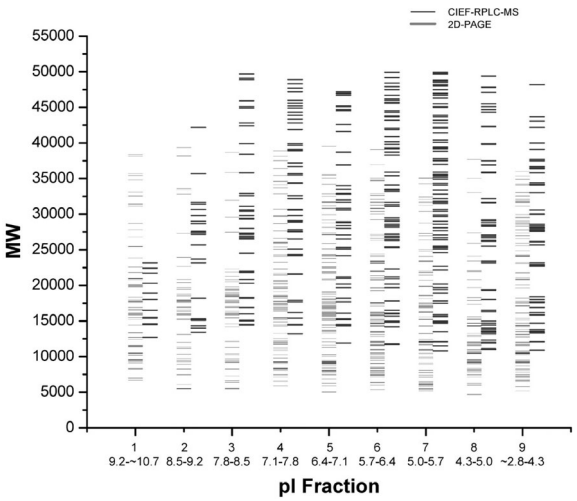

Figure 9. The overlapped p*I* versus MW display of CIEF-RPLC-MS (thin bars) and 2D PAGE (wide bars) data of the intact protein profiling of *Chlorobium tapidum*. Reprinted with permission from [33]. Copyright 2015 American Chemical Society.

CIEF-RPLC-MS (thin bars) and 2D-PAGE (wide bars) in Fig. 9. It can be seen that far more proteins are detected with CIEF-RPLC-MS than 2D-PAGE especially in the low mass and high p*I* range.

In summary, hyphenation of CIEF and LC-MS can be achieved in different ways. In order to ensure the transport of the analytes into the LC system, one key step in the development of a CIEF-LC-MS coupling is to guarantee a current connection to the capillary. This was achieved by a valve, a PEEK tee, or a dialysis membrane. Furthermore, up to this point, all approaches required at least one additional trap column step to store the focused bands prior to the analysis by the LC-MS system and separate them from the ampholytes. This step increases on one hand the analysis time but on the other hand allows a full separation of ampholytes and analytes which ensures a better ionization and higher signal intensity in MS. The following LC analysis gives the possibility for further separation of the analytes due to the orthogonal separation principle to IEF. These advantages made the CIEF-LC-MS system to a high-throughput filter for proteomics research. However, the introduced CIEF-LC-MS system comes with long run times and a complex instrument set up, including different valves, pumps, columns, storage loops, and dialysis membranes.

2.9 Other CIEF–MS approaches

One way to separate two fractions which differ in mass or charge is a free flow electrophoresis (FFE). In order to avoid the negative effect of ampholytes, acids, and bases on the ESI process, an FFE unit was placed between the CIEF separation and the MS by Chartogne et al. [35]. An FFE chamber size of 10 × 25 mm was used and continuously flushed with 1% acetic acid and a flow rate of few microliters per minute. This set up makes it on one hand possible to fully separate the analytes and ampholytes but on the other hand results in poor separation efficiency, loss of sample between focusing and mobilization, and makes it impossible to calculate the exactly injected sample amount due to the counter pressure from the FFE process.

In another experiment, Kim et al. analyzed phosphorylated proteins by CIEF [36]. In order to achieve a successful coupling to the MS system an asymmetric flow field-flow fractionation step was applied. The fractionated samples were injected into a nanoLC system (C18AQ column) hyphenated with an ESI-IT-MS. It was claimed that this high-throughput 2D separation method can provide a viable alternative to the classical 2D-GE (IEF/SDS-PAGE) approach. The isolation of phosphoproteins from a proteomic sample was achieved, which required a narrow pH cut off. However, the separation was based on a complex instrumental setup including a collection step prior to entering to the MS detection. The parameters from Kim et al. can be found in Table 5.

Table 5. Other CIEF–MS approach

Other CIEF-MS approach								
Anolyte and catholyte solution	Interface	MS; Sheath liquid	Focusing	Mobilization	Ampholytes; p*I* range	Application	Additional information	Ref.
1% acetic acid as weak acid and weak base	Chip-based FFE	ESI-Q-MS; 50% methanol, 49% H_2O, and 1% acetic acid (2 μL/min)	25 kV + pressure from inlet	By pressure	5% Pharmalyte (Pharmacia, Uppsala, Sweden); p*I* range: 5–8	Myoglobin (horse heart), carbonic anhydrase I (human erythrocyte) and β-lactoglobulin B (bovine milk)	FFE voltage: 56 V	Chartogne et al., 2000 [35]
CIEF	Asymmetric flow field-flow fractionation and nLC	ESI-IT-MS	Offline	40% Fluka ampholyte high-resolution (Sigma)	3–6	Lysate from prostatic cancer cell line		Kim et al., 2012 [36]

3 Conclusion and future prospects

In conclusion, the combination of CIEF with MS detection provides a powerful tool, especially for protein analysis. CIEF-MS shows some great advantages like a high resolving power, especially for analytes with similar structures, a self-sharpening and analyte stacking effect which can lead to higher sensitivity than CZE-MS (e.g. a concentration factor of 240 has been reported [31]). For proteomic analysis, CIEF can be online coupled to an LC-MS system. With this setup, a high number of proteins could be detected especially in the range smaller than 10 kDa, larger than 40 kDa, at extreme p*I* values and membrane proteins [31, 33]. CIEF-MS shows a greater dynamic range, a higher sensitivity, and larger throughput than achieved with 2D PAGE-MS approaches [31]. In addition, it is possible to work with intact proteins [20]. This is especially interesting in combination with MS/MS experiments like ETD (top-down approach). CIEF-MS is in general more robust than 2DGE coupled to MS [37].

Several approaches have been studied: The most common approaches were performed by direct coupling of CIEF-ESI-MS using low ampholyte concentration in order to reduce negative effects during the ionization process to an acceptable level. On one hand, this resulted in a higher sensitivity of the MS detection, and on the other hand in lower separation efficiency. However, accepting this compromise, it is possible to achieve an online coupling of CIEF and MS leading to comparable short run times and a real alternative to the classical 2D gel followed by LC-MS approach. Another attempt is the coupling of CIEF with LC-ESI-MS. However, this requires often complex and expensive interfaces and results in longer run times. Up to this point, the application of an immobilized pH gradient or the use of the partial filling technique is not applicable for a large range of analytes. FFE as an interface to separate analytes and interfering components provide an interesting approach. As another promising approach in the hyphenation of IEF with MS, it has been suggested to perform IEF in a multiple junction capillary. However, both concepts need to demonstrate their general applicability.

In particular with the direct CIEF-MS coupling, some applications in biopharmaceuticals analysis, medicine, or biochemistry (proteomics) have been demonstrated with great success. Especially in the proteome analysis of cell digests, IEF coupled with MS leap forward the development and shows a real alternative to the classical 2D gel followed by LC-MS approach. This underlines the strong demand in bioanalytical sciences for a highly efficient IEF-MS hyphenation system. So the search for a simple and reliable system which preserves the high resolving power in the focusing process and offers high sensitivity in MS detection continues. This goal is especially of great interest in the context of increasing performance and availability of high-resolution MS and fragmentation options for intact proteins.

We gratefully thank Sabine Neuberger and Kevin Jooß from Aalen University for helpful suggestions during the work on this review.

The authors have declared no conflict of interest.

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Paper II

Anal Bioanal Chem (2016) 408:4055–4061
DOI 10.1007/s00216-016-9498-8

RESEARCH PAPER

CIEF-CZE-MS applying a mechanical valve

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Received: 16 December 2015 / Revised: 14 March 2016 / Accepted: 16 March 2016 / Published online: 6 April 2016

Abstract Separation and determination of proteins by capillary isoelectric focusing (CIEF) and mass spectrometry (MS) are essential and complementary techniques in the field of bioanalysis. The hyphenation of these two techniques is challenging due to the nonvolatile substances required for the CIEF separation. An additional separation step prior to MS enables the removal of the nonvolatile substances. However, it is complicated due to the small transfer volume and the required high voltages in the CIEF process. In order to remove nonvolatile substances and transfer the analytes toward the mass spectrometer, we applied a four-port valve to couple CIEF online to capillary electrophoresis-mass spectrometry. To demonstrate the power of this concept, hemoglobin and glycated hemoglobin with an isoelectric point difference of 0.037 were separated via isoelectric focusing and characterized by MS. In general, this setup guaranties interference-free mass spectra and will provide an information-rich and sensitive top down protein characterization.

Keywords Capillary isoelectric focusing · Mass spectrometry · Online hyphenation · Protein · Two-dimensional separation

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Introduction

Isoelectric focusing (IEF), where amphoteric molecules are separated in accordance to their isoelectric point (p*I*), is a core technique for the separation of proteins and peptides out of complex biological matrices or in common biopharmaceutical samples [1–3]. Isoelectric focusing of molecules can be performed in gel or liquid utilizing a mobile or immobilized pH gradient. IEF is applied to determine the isoelectric point of an analyte, providing characteristic information about that very compound and its quantity after separation from other compounds with different p*I* values.

In order to characterize complex protein samples, IEF in a flat gel is commonly combined with sodium dodecyl sulfate polyacrylamide gel electrophoresis [4]. This technique provides a high separation capability due to the orthogonality of p*I* and mass. However, the technique is associated with certain drawbacks such as highly intense lab work, long analysis time, necessity of a large sample amount, and elaborate off-line coupling to a mass spectrometry (MS) system for accurate mass detection. One approach to overcome these limitations and improve mass accuracy is the hyphenation of capillary-based IEF (CIEF) with high-resolution MS. Isoelectric focusing was first performed in a capillary by Hjertén and Zhu [5]. This important achievement enables the application of IEF in a faster and more reproducible way. However, by coupling CIEF to MS, several issues need to be considered: (i) A high ampholyte concentration, being essential for achieving high separation efficiency in the CIEF analyses, causes interferences in the electrospray ionization (ESI) process leading to suppressed signal intensities in the MS detection [6]. (ii) The involatility of the commonly applied acids and bases, utilized to generate the pH gradient, causes interferences regarding the ESI process [7]. (iii) A small sample amount has to be transferred in a reproducible way without further dilution from the

CIEF separation toward the MS interface. (iv) Two high voltage systems with different current properties have to be handled independently. Taking these issues into consideration, several coupling strategies have been developed [8]. The most commonly applied strategy represents the direct CIEF-MS coupling with low ampholyte concentration [6]. Other groups have been working with liquid chromatography or a dialysis unit as an interface between the CIEF separation and the MS detection [6, 7, 9]. However, maintaining high separation efficiency and a good sensitivity as well as a short analysis time is difficult even with these advanced approaches.

The potential of multidimensional electrophoretic separation was recently highlighted in two analytical reviews [10, 11]. Up to date, a hyphenation of CIEF and an electro-driven separation technique was only demonstrated with UV detection in the second dimension. Of particular interest is the coupling between CIEF and capillary electrochromatography (CEC) applying a mechanical valve, demonstrated by Zhang and El Rassi [12]. In this work, a proof of principle for the coupling of two electro-driven separation techniques using a mechanical valve was shown by the separation of model and human serum proteins. Utilizing a six-port valve and a pressure-driven transfer through the valve, a minimum of 130 nl was transferred as a fraction into the CEC. This represents a suitable injection volume regarding CEC; however, this volume is too large for capillary zone electrophoresis (CZE).

For an interference-free MS analysis of CIEF-separated analytes, we successfully hyphenated a CIEF separation to a capillary zone electrophoresis-mass spectrometry system via a four-port valve, including a 20-nl loop. Utilizing this system, it is possible to transfer focused analytes without further dilution from the CIEF separation into the CZE system while excluding the majority of the ESI interfering substances. The remaining interfering substances are further separated from the analytes by the CZE separation in the second dimension prior to online ESI-MS detection.

Materials and methods

Materials

Ultrapure water with an electrical resistivity >18 MΩ cm was supplied by an UltraClear UV system (Siemens Water Technologies, Günzburg, Germany) and was used for the preparation of all samples, rinsing solutions, and background electrolytes (BGEs). Fused silica capillaries (50 μm i.d., 363 μm o.d.) were obtained from Polymicro Technologies (Phoenix, AZ, USA). Methanol (MS grade), isopropanol (MS grade), and sodium hydroxide (≥98 %) were received from Carl Roth (Karlsruhe, Germany). L-Arginine, myoglobin from equine skeletal muscle (95–100 %), β-lactoglobulin A from bovine milk (≥90 % PAGE), ribonuclease A from bovine pancreas (≥60 % SDS-PAGE), and hemoglobin (human) were obtained from Sigma-Aldrich (Steinheim, Germany). Glycated human hemoglobin (16.1 %) was obtained from Canterbury Scientific Ltd (Christchurch, New Zealand), and hydrochloric acid (37 % p.a.), phosphoric acid (85 % p.a.), and formic acid (99–100 % p.a.) were purchased from Häffner GmbH & Co KG (Asberg, Germany). Iminodiacetic acid (98 % p.a.) was used from Merck (Darmstadt, Germany). UltraTrol™ dynamic Pre-Coat LN was obtained from Target Discovery (Palo Alto, CA, USA). Servalyt 3-10 and 6-8 (40 % *w*/*v* solution in water) were from Serva Electrophoresis GmbH (Heidelberg, Germany). The prototype C4N-4354-.02D microinjector four-port valve with an internal 20-nl sample loop including the rotor and stator out of a polymer material (Valcon E®, polytetrafluorethylene (PTFE)/PEEK composition) was purchased from VICI AG International (Schenkon, Switzerland).

CIEF(UV/C^4D)-CZE-MS

The interference-free MS detection of one CIEF-separated protein per analysis was accomplished by the coupling of one capillary electrophoresis (CE) instrument performing CIEF separation (CE1 in Fig. 1) with a second CE instrument performing CZE-MS (CE2 and MS in Fig. 1). Both CE instruments were connected to a four-port valve in a way that the separation paths of both CE instruments pass through the valve. That setup enables to switch part of the separation path (i.e., the 20-nl loop) from the CIEF into the CZE separation (position A to position B in Fig. 1). Hence, one individual protein band can be transferred from the CIEF into the CZE-MS dimension. The full setup is schematically displayed in Fig. 1. To convey a better understanding of the CIEF separation, the four-port valve and the CZE separation methods are explained individually in detail in the following chapters.

CIEF-UV/C^4D

CIEF with UV and conductivity (C^4D) detection was performed using a Beckman Coulter PA 800 plus capillary electrophoresis instrument (Beckman Coulter, Krefeld, Germany), and 0.01 M phosphoric acid was used as anolyte solution for the separation of the model proteins (equine myoglobin, bovine β-lactoglobulin A, and bovine ribonuclease A). For the analysis of the hemoglobin variants, 0.1 M phosphoric acid was used and 0.02 M sodium hydroxide was utilized as catholyte solution for both applications. One capillary was plugged into junctions S and W at the four-port valve (see Fig. 1.). A 30-cm capillary, with a window at 4.4 cm, was mounted in front of the valve and a 20-cm capillary after the valve. New fused silica capillaries were conditioned by flushing with approximately 1 bar for 5 min with methanol, 5 min

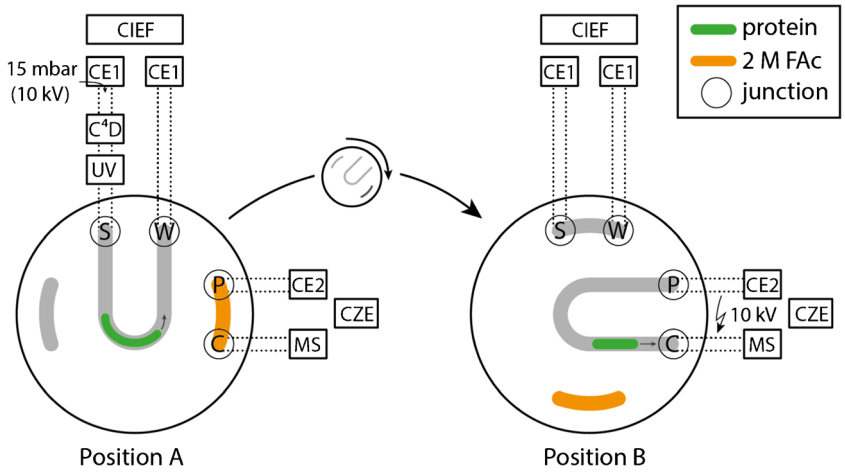

Fig. 1 Scheme of the full 2D system showing CE1 with the CIEF separation including the C^4D and UV detectors connected to junctions S and W of the four-port valve. CE2 and MS which represent the CZE dimension are connected to junctions P and C at the valve. During the CIEF separation and mobilization, the rotor of the valve is in position A until the protein of interest reaches the sample loop. After stopping the CIEF mobilization, the rotor is switched to position B, the CZE separation is switched on, and the protein is migrating toward the MS detection

with water, 20 min with 1 M sodium hydroxide, and 5 min with water. Before each measurement series, an initial coating was performed, including the following steps: flushing with approximately 1 bar with water, 0.1 M hydrochloric acid, water, UltraTrol™ dynamic Pre-Coat LN, and water for 5 min, respectively. Prior to each run, the capillaries were flushed with approximately 1 bar water and phosphoric acid for 4 min each, followed by filling the capillary with sample (400 ppm of each protein, 0.001 M iminodiacetic acid, 0.0214 mM L-arginine, and 1.25 % *w/v* Servalyt 3-10 for the model protein solution and 500 ppm of Hb/Hb_{1C}, 0.9 mM iminodiacetic acid, 0.0234 mM L-arginine, and 1 % Servalyt 6-8 for the hemoglobin variant solution) for 2 min with approximately 1 bar. The focusing step was completed after applying 10 kV over 6 min for the model proteins and 9 min for the hemoglobin variants. Mobilization was performed by pressure (15 mbar) for at least 5 min. In order to keep the self-sharpening effect of the CIEF separation, an additional voltage of 10 kV was applied during the mobilization. Two external detection systems were used in this instrument setup. An external C^4D detector from Innovative Sensor Technologies GmbH (Strasshof, Austria) was placed 6.5 cm in front of the loop of the valve, and an external UV detector from J+M Analytik AG (Essingen, Germany) was positioned 4.95 cm in front of the middle of the valve. The end of the L-arginine zone was detected at 200 nm with the UV detector and the C^4D detector. The model proteins and hemoglobin variants were only detected by the external UV detector at 200 and 420 nm, respectively. The time when the analytes are positioned in the loop of the valve was calculated from the time difference between the detection systems. This calculation is illustrated in detail in Fig. 2. In a first step, the time difference

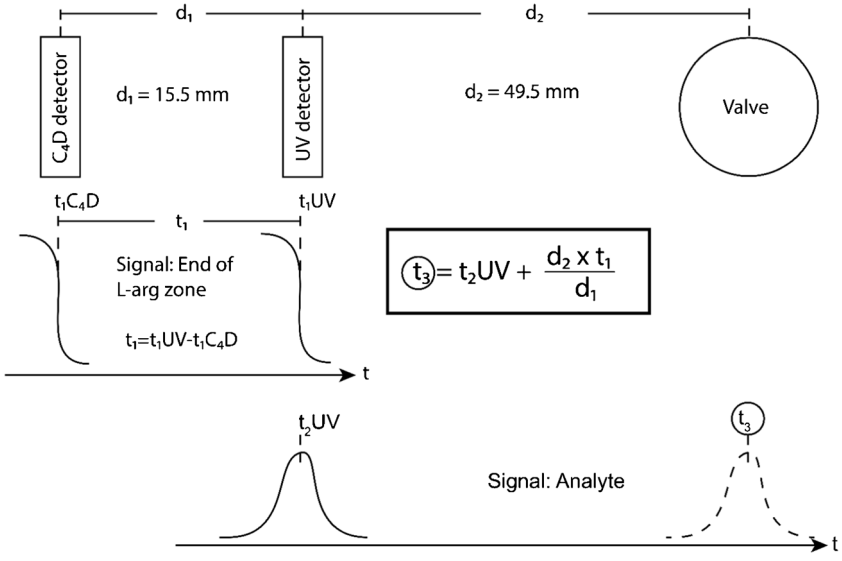

Fig. 2 Calculation of the arriving time (t_3) of the analytes in the loop using the mobilization time difference of the L-arginine zone (t_1) between the two external detectors (conductivity and UV absorption), the distance (d_1) between the C^4D and the UV detector, the distance (d_2) between the UV detector and valve, and the mobilization time (t_2(UV)) of the analyte detected by UV absorption

(t_1 in Fig. 2) of the end of the L-arginine zone between the C^4D and the UV detector was calculated. By applying pressure for the mobilization, L-arginine and the analytes are moving with the same velocity. Knowing the exact distance (d_1) between the C^4D and the UV detector, the distance (d_2) between the UV detector and the middle of the valve, and t_1, it is possible to calculate the time which the analytes require to pass distance d_2. Equation 1 and the mobilization time (t_2(UV)) of the protein enable the calculation of the time (t_3) when the analyte is arriving at the center of the sample loop inside the valve.

$$t_3 = t_2(\mathrm{UV}) + \frac{d_2 * t_1}{d_1} \quad (1)$$

Four-port valve

A prototype four-port valve including a 20-nl loop was placed between the CIEF and the CZE-MS systems as an interface. The four-port valve represents a prototype, and thus, the application for electrophoretic-based separation is not completely evaluated. The valve consists of three parts: a stator with four junctions (S, W, P, and C in Fig. 1) connecting the capillaries with finger tight screws. The other ends of the junctions possess an outlet channel of approximately 50 μm ending at one of the three loops on the rotor. The rotor with the three loops, where the sample loop has a volume of 20 nl, is shown in Fig. 1. The third part of the valve is a motor (not shown in Fig. 1), which enables manual switching between two available positions. During the measurements, all three parts are carefully pushed together with two screws. In order to achieve a flat and clean surface, the stator was polished prior to the first application. The polishing process was performed in-house and included the following steps: first, a prepolishing with abrasive paper (grain size 320/500/1200), followed by a polishing step with a 9-μm UltraPad polish wipe (Buehler, Düsseldorf, Germany), and a final polishing with 3- and 1-μm VerduTex diamond suspension (Buehler, Düsseldorf, Germany). The valve was used to transfer CIEF-focused analytes in a heart cut approach into the CZE-ESI-MS system. Therefore, the focused proteins were mobilized (15 mbar and 10 kV) toward the valve until they reached the loop. At this point, the pressure and voltage were switched off, the anolyte and catholyte vials were removed, the valve was switched, and 10 kV was applied in the second dimension to the CZE system. Thus, the protein was migrating toward the MS system for detection.

CZE-ESI-qTOF-MS

The CZE-MS analysis was performed with an HP ^{3D}CE electrophoresis instrument from Agilent (Agilent Technologies, Waldbronn, Germany) and a micrOTOF-Q instrument from Bruker Daltonics GmbH (Bremen, Germany). The MS instrument was calibrated in the range of 100–2500 *m*/*z* (with maximum on *m*/*z* 1521) by direct infusion of "ESI-L Low Concentration Tuning Mix" from Agilent Technologies at 240 μl/h, and 0.2 M formic acid was used as BGE. One bare fused silica capillary was plugged to junctions P and C of the four-port valve, respectively (see Fig. 1). The inlet part between the CE instrument and valve and the second part between the valve and ESI interface were 52 and 35 cm long, respectively. The 35-cm capillary was placed inside the CE-ESI-MS sprayer in such a way that approximately 0.05 mm of the capillary stuck outside of the sprayer. Isopropanol/water (60:40 *v*/*v*) containing 0.2 M formic acid was used as sheath liquid delivered at a flow rate of 240 μl/h. New capillaries were initially conditioned and coated with UltraTrol LN as described already for the CIEF capillaries. Prior to every analysis, the system was flushed with approximately 1 bar for 5 min with BGE. The CZE analysis of the proteins and Hb variants were performed applying 10 kV for at least 20 min. The MS detection was carried out in positive ionization mode with an end plate offset of −500 V and a capillary voltage of −4500 V. The nebulizer gas pressure was set to 0.2 bar and the dry gas flow to 4.0 l/min with a temperature of 170 °C.

Results and discussion

In order to validate the full 2D setup, an aqueous sample, containing 400 ppm of three model proteins, was mixed with iminodiacetic acid as an anodic spacer, L-arginine as a cathodic spacer, and Servalyt (3-10). An UltraTrol™ Pre-Coat LN coating was selected to prevent any adsorption of proteins at the fused silica capillary wall. An electric osmotic flow (EOF) was not observed during the runs and the measurements were stable for at least 20 runs. The whole first dimension was filled with sample. The proteins were focused and mobilized into the loop of the valve. All three proteins were baseline separated in the UV detector, and the passage of the end of the L-arginine zone was well indicated by the abrupt decrease in signal intensity at the UV and C^4D detectors, which is shown in Fig. 3a. It is evident that the focusing is finished after 5 min because of the plateau reached by the current. During the mobilization, the current was stable or slightly increasing. The current profiles of all 8 runs on 2 days were matching, which substantiates a stable and reproducible setup. The good repeatability of the proteins and the L-arginine zone mobilization can also be seen in Table 1. Relative standard deviation (RSD) values for the mobilization intraday of <4.96 % and interday of <5.78 % were achieved. The reproducibility of the mobilization represents an import factor for a precise transfer of analytes using the sample loop of the four-port valve. It is also an indicator that no blockage was created between different measurement series. A blockage would slow down the

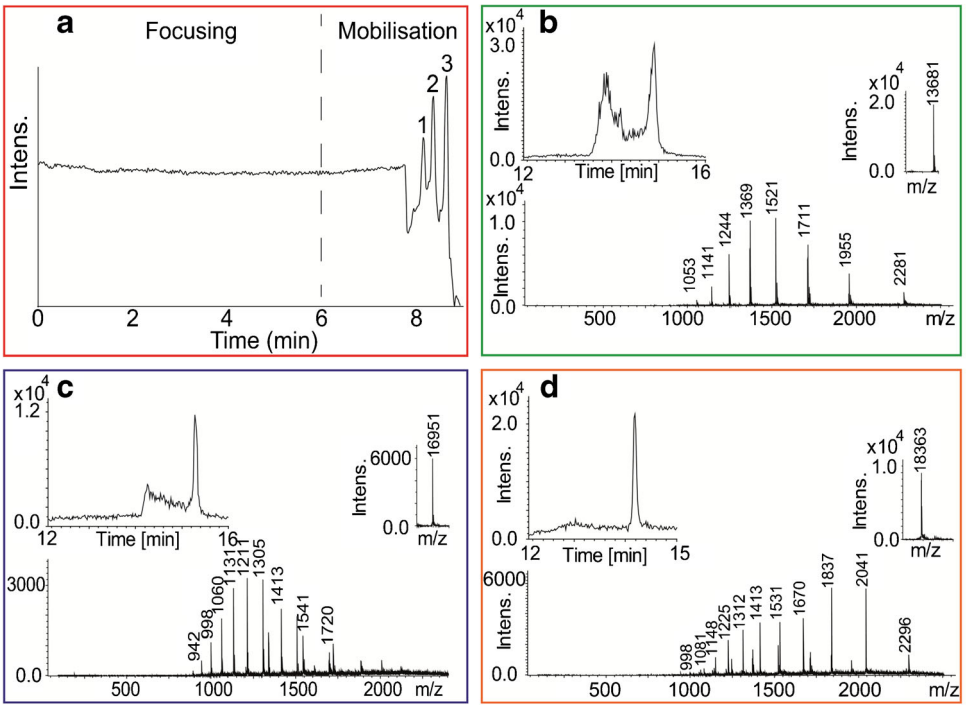

Fig. 3 Electropherogram from the external UV detector at 200 nm (**a**). Abrupt fall of intensity indicates the end of the L-arginine zone (p*I* = 11.2). Signals in the electropherogram of CIEF (**a**): ribonuclease A (p*I* = 9.6) (*1*), myoglobin (p*I* = 6.8–7.2) (*2*), and β-lactoglobulin (p*I* = 5.1) (*3*). Focusing time: 6 min with 10 kV. Mobilization with 15 mbar and high voltage. Catholyte: 0.01 M H_3PO_4; anolyte: NaOH 0.02 M. CZE dimension (background electrolyte, 0.2 M formic acid; 10 kV) with MS detection cutting the peak of ribonuclease A (**b**), myoglobin (**c**), and β-lactoglobulin (**d**) in individual runs, each with base peak electropherogram (*upper left side*), mass spectrum (*lower part*), and deconvoluted mass spectrum (maximum entropy, *upper right side*)

velocity of the mobilization. The transfer time of the proteins from the UV detector to the sample loop was calculated to be 0.75 min (±10.13 %, $n = 4$), which seems to be short enough for a precise transfer of proteins from the first into the second dimension.

For a 2D coupling of electromigrative separation techniques, a fully isolated valve is required. Thus, a polymer-based valve out of polyether ether ketone (PEEK)/PTFE was used. The applied PEEK/PTFE possesses a relatively high dielectric strength of ~20 kV/mm and is chemically inert against most of the commonly applied chemicals for electrophoretic separations. Taking dielectric strength of ~20 kV/mm in combination with the shortest distance between the two electrophoretic separation paths inside the valve allows a voltage in the valve of up to about 15 kV to prevent any current breakthroughs through the valve material. For this reason, a maximum of 10 kV was applied for all CIEF and CZE methods. A discussion about peak broadening, dimensions, and current breakdowns within the four-port valve was recently published by our group [13]. The arriving time of the

Table 1 Mobilization time and the associated relative standard deviation (%) of the end of the L-arginine zone, the three model proteins, hemoglobin, and the glycated hemoglobin. Mobilization values differ between model protein and hemoglobin variants due to the different focusing time

Signal	Detector	Mobilization time (min) in first dimension ±RSD (%)	
		Intraday ($n = 4$)	Interday ($n = 8$)
End of the L-arginine zone (model protein method)	C^4D	7.02 ± 4.65	7.24 ± 5.78
	UV	7.33 ± 2.12	7.59 ± 5.20
Ribonuclease A	UV	7.73 ± 4.96	8.02 ± 5.21
Myoglobin	UV	7.95 ± 4.42	8.25 ± 5.90
β-Lactoglobulin	UV	8.22 ± 2.04	8.51 ± 5.31
End of the L-arginine zone (hemoglobin method)	C^4D	9.15 ± 0.75	
	UV	9.33 ± 0.84	
Hemoglobin	UV	9.86 ± 1.55	
Glycated hemoglobin	UV	10.02 ± 1.05	

proteins at the sample loop of the valve was calculated with Eq. 1. A detailed explanation of the calculation can be found in the method part of this article. For a precise heart cut of one individual protein, the detection should be as close as possible to the sample loop. Ideally, a detection at the sample loop is aspired, which would make it possible to cut out the protein without the need for two detection systems, along with the transfer calculation. In our setup, the closest possible position of the detection in front of the valve was 6.5 cm for the C^4D and 4.95 cm for the UV detector. A second important factor for a precise transfer of the protein from the CIEF separation into the CZE-MS system is the size of the sample loop in comparison with the band volume of the protein during the mobilization in the CIEF dimension. The valve contains a 20-nl sample loop. A band volume of about 12 nl was calculated taking the mean UV band width of the model proteins of 6 s and the transfer time of 0.75 min into consideration. That means that a single CIEF band can be completely transferred to the second dimension. However, the simultaneous transfer of closely focused proteins is possible. Therefore, the use of smaller sample loops will be considered in future experiments.

At the calculated time, when the protein of interest is positioned inside the loop of the valve, the mobilization in the CIEF dimension is stopped and the valve is switched. The protein, which is injected via the loop into the second dimension, is migrating toward the ESI-MS applying 10 kV using a formic acid background electrolyte. The reproducibility of the cutting and the CZE-MS analysis was evaluated by the analysis of β-lactoglobulin in three individual runs. The migration time from the sample loop to the MS detection was measured with 15.93 min ± 3.46 %. The signal intensity at the migration time of β-lactoglobulin for the base peak electropherogram was $2.26 \times 10^4 \pm 5.23$ %. The good reproducibility of the intensity of β-lactoglobulin shows that the transfer of the protein from the CIEF separation into the CZE separation works precisely between different runs. Throughout all runs, a stable current of 5.6 ± 0.2 μA was established in the second dimension. It was possible to detect all three proteins by MS (Fig. 3b–d). For all three proteins, interference-free mass spectra were obtained, without significant signals of co-migrating substances. This is in agreement with the separation of ampholytes and protein, which is shown in Fig. 4b. The mass spectra and the deconvoluted mass of all three proteins are matching with the literature (Fig. 3b–d) [14–16]. The difference between the calculated deconvoluted mass (maximum entropy) from this experiment and the values from the literature was ≤1 ppm. Due to the injection of the sample in the whole capillary, a factor of 33 or higher intensity was achieved compared to CZE-MS with the same sample concentration and injection time of 24 s with 50 mbar. This factor of 33 is in agreement to the estimated factor of 30 of the injection volumes.

In order to demonstrate the power of the proposed valve-based concept, a sample of two analytes which are structurally similar was chosen: hemoglobin (Hb) and the glycated variant of hemoglobin (Hb_{1C}) (p*I* difference 0.037 [17]). For the analysis of the hemoglobin variant, the method from Hempe and Craver [17] was adapted to our system. An aqueous sample of 500 ppm Hb, containing 16.1 % Hb_{1C}, mixed with Servalyt 6-8, L-arginine, and iminodiacetic acid, was analyzed. It was possible to detect the end of the L-arginine zone at 200 nm and the Hb and Hb_{1C} peak at 420 nm in a reproducibly way

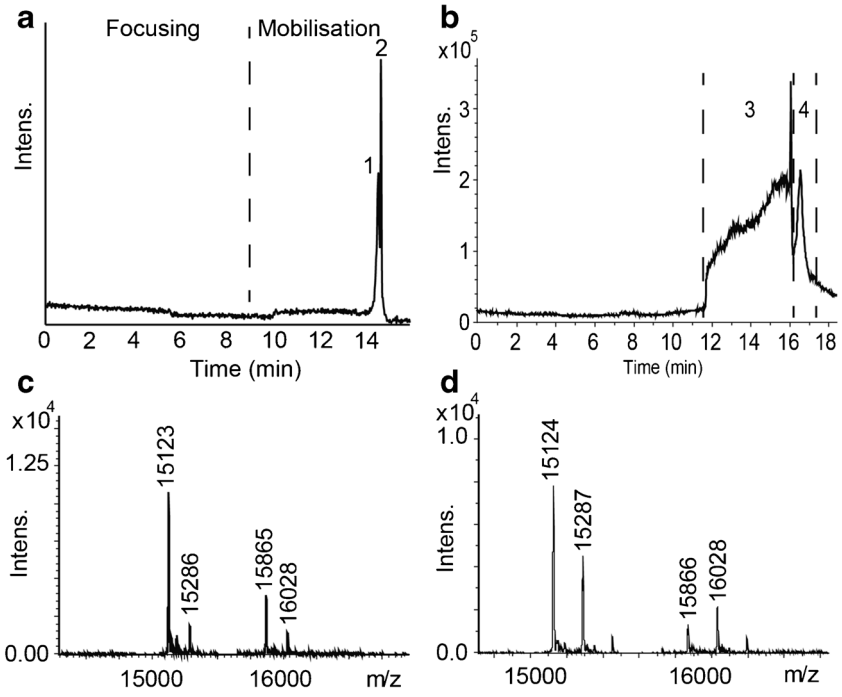

Fig. 4 Electropherogram for CIEF (**a**) of hemoglobin (*1*) and the glycated hemoglobin (*2*) from the external UV detector at 420 nm. Focusing time: 9 min with 10 kV. Mobilization with 15 mbar and high voltage. Catholyte: 0.1 M H_3PO_4; anolyte: NaOH 0.02 M. Base peak electropherogram (**b**) of the second dimension (CZE-MS; BGE: 0.2 M formic acid; 10 kV) showing the separation of ampholytes (*3*) and hemoglobin (*4*). Deconvoluted mass spectra (maximum entropy) of peak 1 (**c**) and peak 2 (**d**)

(Fig. 4a): An intraday ($n=4$) precision with an RSD for the mobilization time of the proteins and the L-arginine zone of <1.55 % was achieved. The detailed reproducibility data are presented in Table 1. The Hb and the Hb_{1C} peak can easily be identified in the electropherogram at 420 nm (Fig. 4a), though not baseline separated. The second dimension separation of ampholyte and hemoglobin was observed to be similar to the model protein system (Fig. 4b). Cutting the Hb peak resulted in a higher relative signal intensity of the nonglycated form, while cutting at the Hb_{1C} position showed a higher signal intensity for the glycated form (Fig. 4c, d). Incomplete individual cutting of Hb and Hb_{1C} can be explained by (i) the already present overlapping of the peaks in the CIEF separation, (ii) an excessive loop size inside the valve, and (iii) a slightly incorrect cutting position. Nevertheless, the Hb/Hb_{1C} example demonstrates the capability to cut and detect peaks in near proximity of each other in the first dimension, particularly showcasing a p*I* difference of only 0.037 p*I* units [17].

Conclusion

Compared with other CIEF-MS coupling strategies, our CIEF-CZE-MS approach shows separation and detection of the focused proteins in a fast and reproducible way. The analytes were sufficiently separated from the ESI interfering substances such as ampholytes, strong bases, and acids without dilution in the interface. The maintenance of the high separation efficiency of (high-resolution) CIEF methods could be successfully demonstrated for our system setup. In addition, the CZE separation bears the capability to achieve further separation. Our approach, applied to complex protein samples, will provide an information-rich analysis of CIEF-separated analytes and is usable with commercially available instrument parts. The system can be utilized for the characterization of unknown signals and protein variants in CIEF separation both in biological and biopharmaceutical applications.

Acknowledgments We gratefully thank Sabine Neuberger and Kevin Jooß from the University of Aalen for helpful suggestions during the work on this paper and Neil R Pattinson (Canterbury Scientific Ltd) for the kind gift of glycated hemoglobin. With regard to the polishing process of the valve, we gratefully thank Elvira Reiter, Department of Material Research, University of Aalen, Germany. We thank an anonymous reviewer for numerous valuable suggestions.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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Paper III

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Received October 8, 2016
Revised November 21, 2016
Accepted November 21, 2016

Research Article

Interference-free mass spectrometric detection of capillary isoelectric focused proteins, including charge variants of a model monoclonal antibody

CIEF represents an elegant technique especially for the separation of structural similar analytes, whereas MS is a state-of-the-art instrumentation for the identification and characterization of biomolecules. The combination of both techniques can be realized by hyphenating CIEF with CZE-ESI-MS applying a mechanical valve. During the CZE step, the remaining ESI-interfering components of the CIEF electrolyte are separated from the analytes prior to MS detection. In this work, a multiple heart-cut approach is presented expanding our previous single heart-cut concept resulting in a dramatical reduction of analysis time. Moreover, different sample transfer loop volumes are systematically compared and discussed in regard to peak width and transfer efficiency. With this major enhancement, model proteins (1.63–9.75 mg/L), covering a wide pI range (5–10), and charge variants from a deglycosylated model antibody were analyzed on intact level. The promising CIEF-CZE-MS setup is expected to be applicable in different bioanalytical fields, e.g. for the fast and information rich characterization of therapeutic antibodies.

Keywords:
Isoelectric focusing / Mass spectrometry / Monoclonal antibody / Online hyphenation
DOI 10.1002/elps.201600457

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1 Introduction

IEF combined with SDS PAGE represents a common technique for the characterization of proteins [1]. With this approach, impressive results were achieved based on the orthogonality of pI value and molecule mass. However, the mass accuracy applying SDS PAGE is low and in comparison to MS no additional information by MS/MS experiments can be collected. CIEF, developed by Hjerten and Zhu [2], is a method with reasonable success for the separation and analysis of proteins and peptides [3], providing the pI as an important identification feature. Moreover, CIEF shows a higher accuracy, improved reproducibility and the possibility for quantification and simple automation compared with classical IEF [4]. In order to bring the combination of separation according to the pI and characterization by mass to the next level, a combination of CIEF and MS is aspired unifying the best possible results of both techniques.

Up to date, the most common approach for the hyphenation of CIEF and MS represents the direct coupling via an ESI interface, which was first published by Tang et al. [5] and further developed by Zhong et al. [6]. Following this straightforward arrangement promising results have been achieved, e.g. the analysis of bacteria cell lysates [7] or the characterization of highly basic cytokine human interferon-gamma [9]. Nevertheless, in this way no separation of analytes and highly ESI-interfering ampholytes is achieved prior to MS detection, which inevitably results in considerable signal suppression and loss of data quality. In order to counteract these effects lower concentrations of ampholytes are commonly applied [8]. However, this approach is associated with the deterioration of the CIEF separation efficiency. On the other hand, a variety of applications were performed implementing an HPLC system between CIEF separation and MS detection, e.g. a protein profiling method including an online digestion [10]. In this way, it is possible to fully separate analytes and ampholytes prior to MS detection. However, this

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Abbreviations: IDA, imminodiacetic acid; **PEEK**, polyether ether ketone

hyphenation technique is often associated with a complex and expensive instrument setup, dilution effects in the HPLC, a loss of resolution caused by implemented trap columns, excessive sample loop volumes and long analysis times. Besides the aforementioned limitations both, the direct hyphenation and the application of an HPLC intersystem, are fully comprehensive. Further important CIEF-MS hyphenation techniques have been reviewed recently [11].

MAbs represent the most rapidly growing class of therapeutics in recent years [12]. A variety of translation and stress-induced variants possibly occur during manufacturing and storage, which can have a significant influence on the mAb bioactivity [13]. For this reason, there is a high demand for the analysis of mAbs regarding purity and stability [14]. Traditional analysis technique includes i.a. gel-based IEF and IEC. Analyses based on IEC suffer from long method development times and low resolution, whilst IEF is associated with low throughput and difficulties regarding quantitative information [15]. In contrast, CIEF separations feature high resolution, short method development and analysis times, minimal solvent consumption and concentration factors of up to 500 [3, 12, 16]. However, CIEF methods have to fulfill certain criteria to be considered valid for mAb analysis, including i.a. a minimal EOF combined with a reproducible system [14]. Nevertheless, CIEF alone cannot be applied for complete identification of mAb variants, due to the lack of information associated with the commonly applied optical detection [16]. Therefore, the hyphenation of CIEF and MS would provide a powerful tool for the separation and detailed characterization of mAb charge variants.

In our previous work [17], we have shown a proof of concept study of a single heart-cut CIEF-CZE-MS system using a mechanical valve (20 nL sample loop) for the separation and interference-free MS detection of three model proteins. Here, the pI range of the CIEF dimension was expanded by analyzing a peptide/protein mix comprising six different analytes. The analysis time was significantly reduced by the implementation of a multiple heart-cut analysis of all (six) analytes from one CIEF run. Furthermore, different sample loop sizes were tested and evaluated in order to find appropriate transfer volumes. The applicability of our system to relevant biological samples is demonstrated by the analysis of charge variants of a model mAb.

2 Materials and methods

2.1 Materials

Ultrapure water of an electrical resistivity > 18 MΩcm was supplied by an UltraClear ultra violet (UV) system (Siemens water technologies, Günzburg, Germany) and was used for the preparation of all samples, rinsing solutions, and BGEs. Bare fused silica capillaries (50 μm i.d., 363 μm o.d.) were obtained from Polymicro Technologies (Phoenix, AZ, USA). Methanol (LC-MS grade), isopropanol (LC-MS grade), sodium hydroxide (≥ 98%), acetic acid (supra-quality, 100%) and ammonia bicarbonate (≥ 99%, p.a.) were received from Carl Roth (Karlsruhe, Germany). L-arginine, poly(vinyl alcohol) (≥ 99%, average MW 89 000–98 000), myoglobin from equine skeletal muscle (95–100%), glutaraldehyde solution (50% in water, suitable for photographic applications), ß-lactoglobulin A from bovine milk (≥ 90% PAGE), ribonuclease A from bovine pancreas (≥ 60% SDS PAGE), angiotensin I, human (≥ 93%), carboanhydrase A from bovine erythrocytes (≥ 95%) and cytochrome C from equine heart (≥ 95%) were obtained from Sigma Aldrich (Steinheim, Germany). Hydrochloric acid (37% p.a.), phosphoric acid (85% p.a.) and formic acid (99–100% p.a.) were purchased from Häffner GmbH & Co KG (Asberg, Germany). Imminodiacetic acid (i) (98% p.a.) was received from Merck (Darmstadt, Germany). Servalyt™ 3–10 and 6–8 (40% w/v solution in water) were obtained from Serva Electrophoresis GmbH (Heidelberg, Germany). *N*-glycosidase F was purchased from Roche Diagnostics GmbH (Mannheim, Germany). The C4N-4354-.02D microinjector four-port-valve including rotors (20, 10 and 4 nL sample loop) and stators out of plastic material (Valcon E®, PTFE/polyether ether ketone (PEEK) composition) were purchased from VICI AG International (Schenkon, Swiss). Nanosep 10 K omega centrifugal devices were obtained from Pall Life Sciences (Ann Arbor, MI, USA).

All used capillaries were neutral precoated with PVA to prevent any protein adsorption at the capillary wall and eliminate the EOF. The model antibody (mAb X) sample was deglycosylated by using *N*-glycosidase F. A detailed description of the capillary coating and mAb deglycosylation procedures can be found in the supporting information.

2.2 CIEF-CZE-MS setup

A scheme of the instrumental setup is shown in Fig. 1. In general, the CIEF-CZE-MS methods can be divided into two parts: First, the CIEF separation until the positioning of the analyte in the sample loop of the valve is performed. After switching the valve, the ESI-interfering components are separated from the analyte in the second dimension (CZE-MS). The most important method parameters are presented in the following two sections. An extensive description of the parameters is illustrated in Supporting Information Table S1.

2.3 Part 1: CIEF dimension and positioning of analytes in sample loop

CIEF with an external UV detector (J+M Analytik AG (Essingen, Germany)) was performed as first dimension utilizing an HP ^{3D}CE electrophoresis instrument from Agilent (Agilent Technologies, Waldbronn, Germany). A 40.0 cm PVA coated capillary was connected from CE1 to junction "S" of the mechanical valve through the external UV detector, which is displayed in Fig. 1. A 20.0 cm PVA capillary was connected from junction "W" to an external outlet vial. Thus, the CIEF separation takes place in a total length of 60.0 cm (plus the

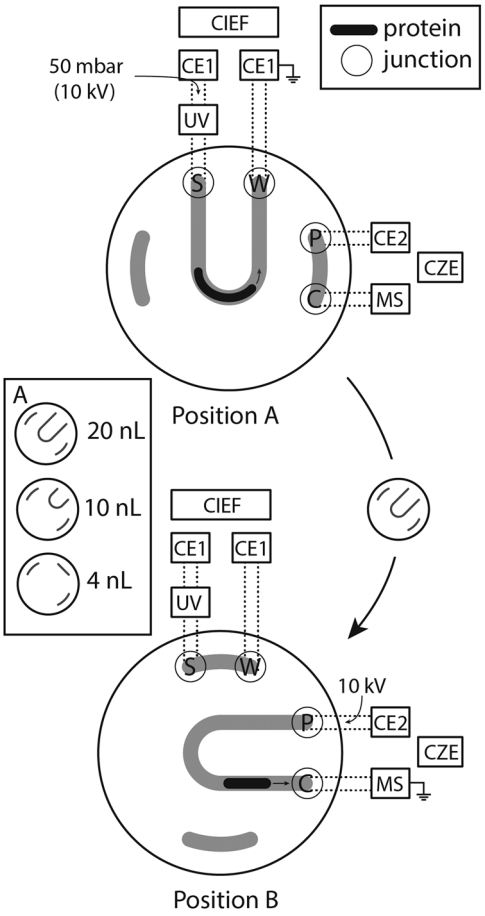

Figure 1. General arrangement of the 2D CIEF-CZE-MS setup. Thereby, CE1 and the UV detector represent the CIEF dimension and CE2 and the MS the CZE dimension. The separation capillaries of the CIEF dimension are connected to junction S and W, whereas the capillaries of the CZE separation are connected to junction P and C. During the mobilization step (with remaining 10 kV to keep the focusing) of the CIEF process the analyte of interest is positioned in the sample loop, subsequently the rotor is switched from position A to position B, connecting the sample loop with the second dimension. Finally, high voltage (10 kV) is applied in the CZE dimension and the analyte migrates to the MS. Inset A shows the appearance of the rotors with the different sample loop volumes which were tested in the here presented work (4, 10 and 20 nL).

distance inside the valve). The focusing distance (prior to UV detection) was 35.5 cm. L-arginine was used to block the distance from UV through the valve to the outlet vial in such a way, that all analytes are focused in front of the external UV detector. Anolyte and catholyte solution vials were kept at the same height to prevent any undesired mobilization by hydrodynamic effects during experiments. The preconditioning procedure prior to every measurement series and run can be found in Supporting Information Table S1. The sample composition is shown in detail in Supporting Information Table S1. Hence, IDA was used to prevent a cathodic drift of the sample, L-arginine was utilized as an anolytic spacer, urea to prevent protein precipitation and Servalyt™ for applying the pH-gradient inside the capillary and the protein, peptide or antibody. Injection was performed with approximately 1.5 bar for 1.5 min. 10 kV were applied for 10 min as focusing step. The mobilization was performed with pressure (50 mbar) and an additional high voltage (+10 kV) to preserve the separation until the signal of interest is positioned inside the sample loop. Therefore, the determination of the flow rate in the 1st dimension prior to each sample injection was required in order to achieve an accurate positioning of the analyte inside the sample loop. Hence, a caffeine standard (1000 mg/L) was injected (50 mbar, 12 sec) and 50 mbar were applied until the caffeine signal appears in the UV detector. Subsequently, the flow rate (50 mbar) was calculated by dividing the volume from the inlet to the UV detector by the required time of caffeine to pass the detector. After the desired analyte was positioned in the sample loop the valve was switched to position B. For the multiple heart-cut approach, 50 mbar were applied for the time distance between the previous and the next analyte in the UV detector, in order to cut and analyze the following analyte species. Cleaning and storage procedures of the capillaries are described in Supporting Information Table S1.

2.4 Part 2: CZE-MS dimension after positioning of the analyte inside the sample loop

CZE-MS was performed utilizing an HP 3DCE electrophoresis instrument (Agilent Technologies, Waldbronn, Germany) and a *compact* quadrupole–quadrupole time of flight (QqTOF) instrument from Bruker Daltonik (Bremen, Germany). Two 40.0 cm long PVA capillaries were connected to the mechanical four-port valve. One was mounted between CE2 and junction "P" and the other between junction "C" and the ESI sheath liquid sprayer of the qTOF MS instrument (see also Fig. 1). Due to the bulkiness of the two CE and the MS instrument, a minimum length of 40 cm was required for the connection of CE2 and junction "P" of the four-port valve. The PVA capillary was positioned in the sheath liquid sprayer in such a way, that about 0.05 mm stuck outside of the sprayer tip. Isopropanol:water (1:1) with 1% (v/v) acetic acid was used as sheath liquid. The preconditioning, cleaning and storage procedures for the capillaries of the CZE dimension is explained in Supporting Information Table S1. The CZE separation was performed in 0.2 M formic acid as BGE applying 10 kV. A detailed description of the MS parameters for each analyte can be found in the supplementary information (Supporting Information Table S1).

3 Results and discussion

3.1 Transferring analyte from CIEF into CZE using a four-port valve

The transfer of the focused analytes toward the CZE-MS dimension represents a crucial step in the 2D system. A scheme

of the transfer process is illustrated in Fig. 1. In our previous work, the analyte positioning procedure was performed with an external conductivity (C^4D) and UV detector [17]. However, the placement of two detection systems in front of the valve and the detection of the L-arginine zone is technical challenging. Therefore, a simpler, more robust, approach was pursued. Hence, the calculation of the positioning time of the analyte inside the sample loop of the valve is based on the application of one detector (UV) and the determination of the flow rate (50 mbar) prior to each analysis. The flow rate was determined with a caffeine standard (1000 mg/L) and 50 mbar, as explained in detail in the method section. At the point when the analyte reached the external UV detector the mobilization (50 mbar, +10 kV for refocusing) was remained for approximately 1 min, based on the calculated flow rate, to position the analyte inside the sample loop. The moment when the analyte has arrived at the sample loop the mobilization was stopped and the valve was switched from position A to B as illustrated in Fig. 1. By the determination of the flow rate prior to each run, minor changes in the flow conditions, e.g. caused by switching the valve, could be compensated to ensure a precise as possible cutting procedure.

3.2 Selection of optimal sample/transfer loop size

Three different sample loop sizes (4, 10 and 20 nL), as displayed in Fig. 1 were tested in this study. In general, the selection of the sample loop size is heavily dependent on the peak widths and resolution of analytes within the CIEF separation. For instance, the 10 nL sample loop size was found to be appropriated for the analysis of the model proteins (concentration: 6.5 to 39 mg/L), which is substantiated by the electropherogram in Supporting Information Fig. S1A. Here, a peak width of 8 (0.09 min) to 11 nL (0.13 min) was calculated taking the flow rate and the UV electropherogram of the mobilized CIEF separation in consideration. The distance of the closest signals, from peak maximum to maximum, was calculated to be 11 nL (0.13 min). In this regard, the 20 nL sample loop did not allow cuts of individual protein species, due to the excessive volume compared with the resolution of the signals. Applying the 4 nL sample loop a very precise cutting of single peaks was possible, however, a significant loss of signal intensity in the MS was consequently observed. In conclusion, the best choice for this application was the 10 nL sample loop, which allows the individual cutting of the analytes combined with the transfer of a majority of the desired analyte species to the 2nd dimension. Nevertheless, if demanded, it is possible to cut more than one analyte, e.g. angiotensin I and myoglobin (signal 1 and 2 in Supporting Information Fig. S1A). Hence, both signals are transferred to the CZE-MS dimension by switching the valve. Thereby, angiotensin I and myoglobin could be baseline separated in the CZE dimension and thus, analyzed individually in the MS, which is shown in Supporting Information Fig. S1C.

Furthermore, the three rotors were tested for the analysis of a model antibody (mAb X). The results are displayed in Supporting Information Fig. S1B. By applying the 4 nL sample loop and cutting on signal 1 peak areas of $4.2\times10^3 \pm 7.9\%$ ($n = 3$) and peak heights of $5.4\times10^2 \pm 11.1\%$ ($n = 3$) were determined by integrating the base peak electropherogram. Despite the successful detection of a signal for mAb X, the sensitivity was too low for an accurate deconvolution which is essential to achieve precise mass determination. Utilizing the 10 nL sample loop peak areas of $8.1\times10^3 \pm 7.7\%$ ($n = 3$) and a height of $8.9\times10^2 \pm 3.1\%$ ($n = 3$), which was sufficient for deconvolution. The application of the 20 nL sample loop resulted in the highest achieved peak areas of $3.4\times10^4 \pm 6.7\%$ ($n = 3$) and peak heights of $1.5\times10^3 \pm 4.0\%$ ($n = 3$). However, the deconvolution resulted in shifting average masses (data not shown), which can be explained by the cut of more than one mAb variant due to the excessive transfer volume. In conclusion, the 10 nL rotor was also the best choice for the analysis of mAb X and thus, used in all further experiments.

3.3 Multiple heart-cut CIEF-CZE-MS of model proteins

In order to significantly reduce the overall analysis time combined with less sample consumption, the heart-cut procedure, described in our previous work [17], was expanded to a "multiple heart-cut" approach, enabling to cut more than one peak within one CIEF run. Therefore, a standard mixture containing five model proteins and one peptide, covering a broad pI range (5.1–10.25), was analyzed. All six signals were baseline separated in the CIEF-UV dimension, as displayed in Fig. 2. Each analyte, from cytochrome C (pI = 10.25) to ß-lactoglobulin (pI = 5.1), was positioned in the sample loop, transferred to the second dimension and analyzed via MS consecutively. Each protein could be identified by their respective MS spectra (see Fig. 2) and the according deconvoluted mass. A separation of analyte and ampholyte molecules for an interference-free MS detection of the CIEF separated proteins was achieved in the same way as demonstrated in our previous work [17]. For a precise transfer of the proteins, a reproducible mobilization time and moreover, a consistent resolution between the individual analyte signals is crucial. Therefore, four UV runs on two different days ($n = 4$) were performed and compared regarding mobilization time. The results are summarized in Supporting Information Table S2. The RSD values of the mobilization time were $\leq 1.86\%$ for all investigated analyte species, which is sufficient for a precise positioning of the analyte inside the sample loop of the valve. Furthermore, the current from start (10.27 µA ± 5.38%) to the end of the focusing step (5.93 µA ± 5.15%) was comparable for all runs, which indicates a reproducible and stable CIEF system setup. No considerable peak broadening of the focused proteins in the CIEF dimension during the individual CZE analyses was observed, which was tested by inverse mobilization of the proteins back to the front of the external UV detector and comparison of the resulting peak widths. In this way, the precise cutting of more than one protein per run was verified. An unintentional mobilization of the focused

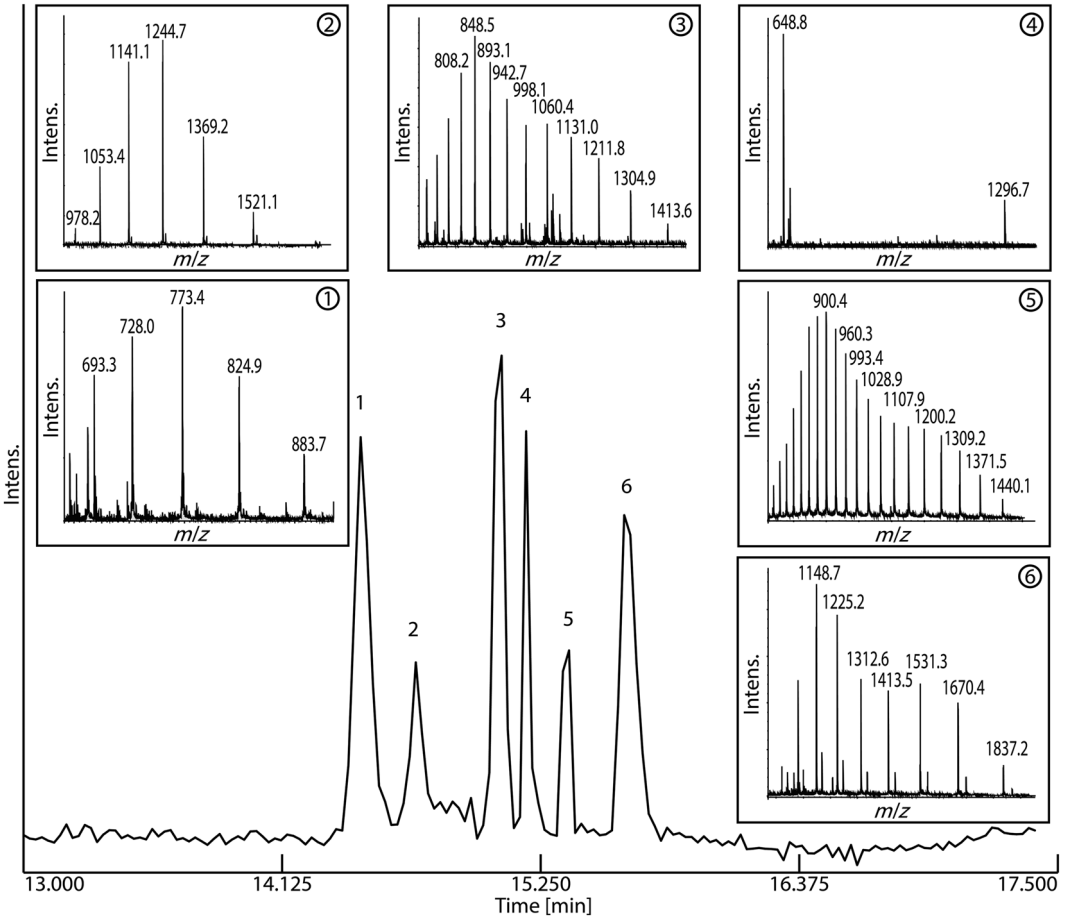

Figure 2. Mobilization of the focused model proteins applying 0.1 M H_3PO_4 as anolyte and 0.2 M NaOH as a catholyte. Focusing was achieved by 10 kV for 10 min. Mobilization was performed utilizing 50 mbar until the first signal is positioned in the four-port valve. The high voltage was remained during the mobilization. UV signals at 280 nm (main Figure) and the MS spectra (inset 1 – 6) at the migration time of the protein in the CZE-ESI-MS dimension are shown: cytochrome C; pI = 10.3 (1), RNAse A; pI = 9.6 (2), angiotensin I; pI = 7.9 (3), myoglobin; pI = 7.2 (4), carboanhydrase A; pI = 6.4 (5) and ß-lactoglobulin; pI = 5.1 (6). The linearity between the mobilization time and the pI value was given ($R^2 = 0.996$).

proteins during the analysis in the second dimension was prevented by adjusting the inlet and outlet vial to the exact same height, avoiding hydrostatic effects. A peak broadening of the focused analytes in the first dimension is noticeable after approximately 10 CZE-MS analyses (~10 min per run). Therefore, a full comprehensive approach was not feasible. Nevertheless, the overall analysis time, characterizing each peak once ($n = 6$), was significantly reduced from >6 h to 2.8 h applying the novel multiple heart-cut approach.

3.4 Multiple heart-cut CIEF-CZE-MS of mAb X

The separation of intact mAb charge variants represents a common application for CIEF, e.g. in product stability testing. Nevertheless, an MS analysis of CIEF separated variants is challenging due to the highly ESI-interfering ampholytes, the closely related structures of these variants and the low volumes in capillary-based separations. Still, the MS analysis of antibody variants is of high interest, especially in the pharmaceutical industry. Therefore, deglycosylated mAb X (solved in formulation buffer) was analyzed as model monoclonal antibody to test the applicability of the 2D system for mAb characterization. Four different signals were observed in the electropherogram of the first dimension after the focusing step, which is displayed in Supporting Information Fig. S2. Signal 1 and 4 were transferred to the second dimension using the multiple heart-cut procedures for further mass spectrometric characterization. The raw and deconvoluted mass spectra of both species can be found in Fig. 3. The deconvolution of signal 1 resulted in an exact mass of 145168.6 ± 0.4 Da ($n = 4$). Signal 4 resulted in an exact mass of 145 170.7 ± 0.6 Da ($n = 4$). A mass difference of 2 Da in combination with a significant shift in the pI value of the variants could be explained by potential deamidation (or amidation) processes. A detailed interpretation of the relative intensity of these variants was not possible due to the occurrence of additional chemical deamidation processes caused

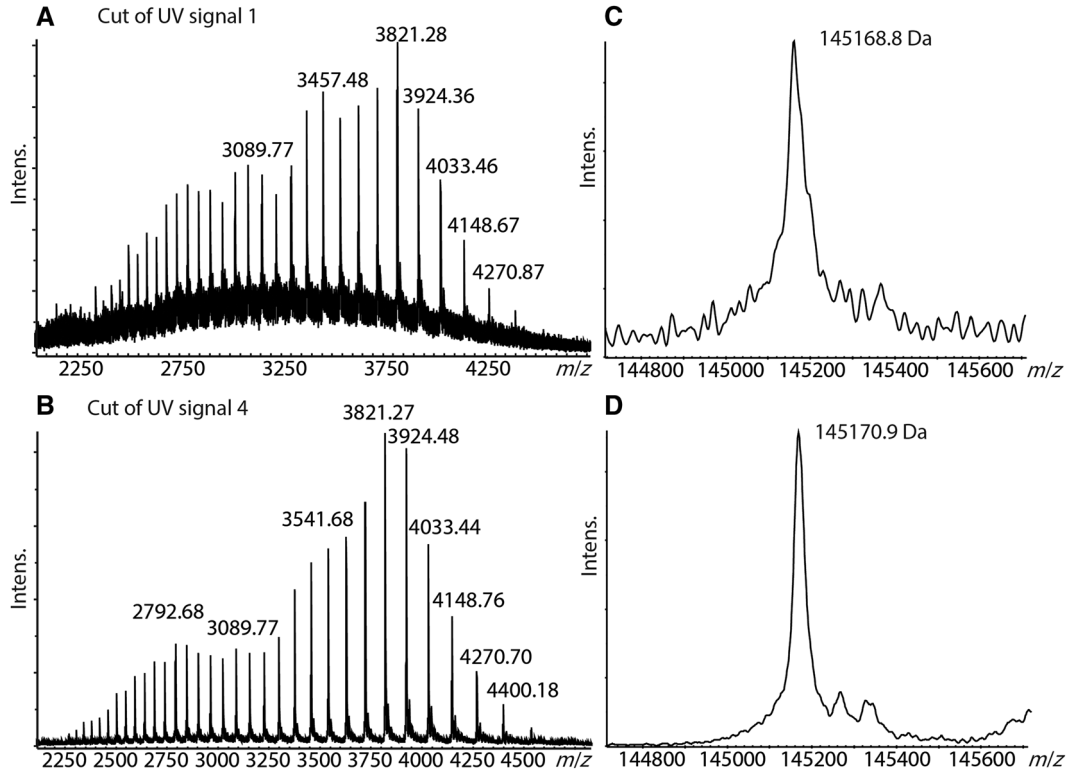

Figure 3. MS identification of variants from CIEF-CZE-MS separation of mAb X. Cuts from focusing of signal 1 (A) and 4 (B) and the corresponding deconvoluted mass spectra (C and D). A difference of approximately 2 Da was observed between the mAb variants.

by the PNGase F treatment [18]. Complementary techniques, such as analyses on peptide level, could support to ensure the occurrence of deamidation products.

3.5 CIEF-CZE-MS limit of detection for proteins

In general, CIEF provides a considerable enrichment factor in comparison to other electromigrative techniques such as CZE, due the IEF separation mechanism and thus, represents an excellent technique for the analysis of low concentrated samples [19]. Therefore, the LOD (S/N 3:1) for the 2D CIEF-CZE-MS approach was estimated. In the case of the low mass (10–50 kDa) protein/peptide mixture the external UV detector proved to be the limiting factor. The LOD of the UV detection (280 nm wavelength) was between 9.75 (RNAse A) and 1.63 mg/L (myoglobin). The UV signal of RNAse A (1) and myoglobin (2) at the LOD is shown in Fig. 4. Obviously, a lower concentration would result in difficulties regarding the clear identification of the protein signals and precise cutting of the individual analytes species. Nevertheless, the lowest concentration (9.75 mg/L for RNAse A and 1.63 mg/L for myoglobin) lead to mass spectra with sufficient intensity for deconvolution. Moreover, the LOD in the MS was estimated to be 0.24 mg/L for RNAse A and 0.20 mg/L for myoglobin. The respective mass spectra of RNAse A and myoglobin at the LOD level of the UV detector are displayed in 4 and C. Reproducibility data of three different concentration levels and two different proteins (RNAse A and myoglobin) are shown in Supporting Information Table S3. The concentrations were selected to cover a range from the model protein experiment (see above) down to the LOD of the protein in the UV detection. As an indicator for a precise cutting, the peak area values of the extracted ion electropherogram of the most abundant charge variant of each protein were considered. As expected, the RSD increased from the highest (5.5–7.9%) to the lowest concentration (17.3–21.6%) due to less signal intensity and difficulties during the identification of the peak maximum in UV for the cutting. Still, a more sensitive detection in the first dimension would enable to analyze even lower concentrated proteins. Nevertheless, the MS identification of proteins in the 1–10 mg/L range represents a promising result.

In the case of the model antibody mAb X, the MS showed to be the limiting factor regarding sensitivity. Appropriate raw mass spectra were achieved for a sample containing 375 mg/L. Still, in order to achieve sufficient signal quality for an accurate deconvolution, a concentration of 1500 mg/L mAb X was required. This rather high LOD can be explained by the low ionization efficiency of high-molecular-weight species such as the analyzed model antibody. Nevertheless, 1500 mg/L for an MS analysis represents an excellent value compared to previous works regarding CZE-MS analysis of antibodies. Exemplarily, Biacchi et al., 2015 demonstrated a glycoform separation and characterization by an offline coupling of CZE-UV with ESI-MS with a sample concentration of 5000 mg/L [20]. Another common top-down analysis

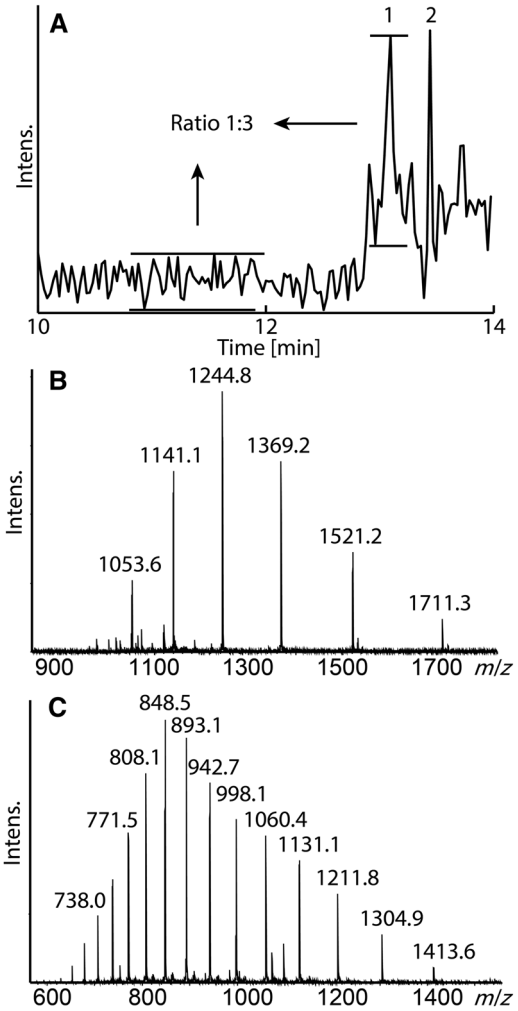

Figure 4. (A) UV signal of CIEF mobilization of a sample containing RNAse A and myoglobin at the LOD (S/N 3:1) level of UV detection (9.75 and 1.63 mg/L, respectively). Noise was defined as baseline noise in proximity to the protein signals. B and C illustrate the respective mass spectra of RNAse A and myoglobin.

technique for mAbs is provided by ultra-high-performance liquid chromatography (UHPLC)-MS. Applying this approach for the analysis of free thiol variants of a recombinant mAb, Liu et al., 2015 analyzed a sample containing 2000 mg/L [21]. Performing an in-house comparison of our CIEF-CZE-MS setup including the 10 nL transfer loop with a CZE-MS system utilizing 10 nL as injection volume, areas of 1.1×10^5 (1500 mg/L) and 0.9×10^5 (30 000 mg/L) were found, respectively. Taking the different concentrations into account a factor of 23 higher sensitivity for the CIEF-CZE-MS setup was determined.

4 Concluding remarks

The high resolution and enrichment factor of CIEF combined with interference-free, high accuracy mass detection represents a promising approach for the analysis of different bioanalytical and biopharmaceutical samples. Compared to our previous work, a "multiple heart-cut" procedure was established considerably reducing analysis time. Up to six analytes, covering a wide pI range of 5.1–10.3, were transferred from a single CIEF run and analyzed via CZE-MS in less than 3 h. In addition, it was proven that it is possible to separate proteins with similar pI values (angiotensin I and myoglobin) in the CZE dimension prior to MS detection. In the case of low to medium molecular weight proteins (10–50 kDa), the external UV detection represents the limiting factor regarding LODs. Nevertheless, it was possible to detect these peptides/proteins down to a concentration of 1–10 mg/L with more than sufficient signal intensity in the MS. Moreover, a more sensitive external UV detector would improve the LOD values of the complete 2D system for these analyte species. Nevertheless, the LOD values indicate the great benefit of the CIEF-CZE-MS approach compared with the classical CZE-MS analysis of proteins. A promising application of the CIEF-CZE-MS system is provided by the mass spectrometric characterization of intact mAb charge variants. It was possible to detect a mass difference of approximately 2 Da between two variants of mAb X. In contrast to the MS analysis of antibody variants by CIEF followed by fraction collection combined with HPLC-MS, the here presented system represents a straight forward and fast approach. The CIEF-CZE-MS setup using a mechanical valve embodies a highly promising system, which can potentially be applied in many different fields of pharmaceutical and biochemical analysis, such as the demonstrated characterization of mAb charge variants.

The authors have declared no conflict of interest.

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Paper IV

Two-dimensional capillary zone electrophoresis mass spectrometry for the characterization of intact monoclonal antibody charge variants including deamidation products

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Submitted to Analytical Chemistry (27.02.2017)

Abbreviations: background electrolyte (BGE), capillary electrophoresis (CE), capillary gel electrophoresis (CGE), capillary isoelectric focusing (CIEF), capillary zone electrophoresis (CZE), ε-aminocaproic acid (EACA), electrospray ionization (ESI), external vial (EV), formic acid (FAc), fused silica (FS), full width at half maximum (FWHM), acetic acid (HAc), hydrochloric acid (HCl), hydrophobic interaction chromatography (HIC), (hydroxypropyl) methyl cellulose (HPMC), inner diameter (ID), ion exchange chromatography (IEX), isopropanol (IPA), monoclonal antibody (mAb), methanol (MeOH), mass spectrometry (MS), sodium hydroxide (NaOH), post translational modification (PTM), polyvinyl alcohol (PVA), quadrupole – quadrupole time of flight (QqTOF), reversed phase liquid chromatography (RPLC), relative standard deviation (RSD), sheath liquid (SL), triethylentetraamine (TETA), tuning mix (TM)

Keywords: antibody analysis, ε-aminocaproic acid electrospray-ionization, capillary zone electrophoresis-mass spectrometry, 2D interface, mechanical valve

Total number of words including figure and table legends:

Abstract

Capillary zone electrophoresis (CZE) represents a powerful tool which is progressively applied for the separation of monoclonal antibody (mAb) charge variants. However, characterization of these variants by mass spectrometry (MS) is challenging, due to the necessity of separation electrolytes which are in many cases highly interfering with the electrospray ionization process. Here, a heart-cut CZE-CZE-MS setup is presented for detailed mAb characterization, using a generic ε-aminocaproic acid based background electrolyte as 1st dimension and acetic acid as 2nd dimension. A hyphenation of two electrophoretic separation systems premises a fully electric isolated interface, small transfer volumes and low-dead-volume capillary connections, which was fulfilled by implementing a mechanical 4-port valve interface. Interference-free, highly precise mass data (deviation < 1 Da) of charge variants of Trastuzumab, acting as model system, were achieved. The obtained mass accuracy (lower ppm range) is discussed regarding both, measured and calculated masses. Deamidation was detected for the intact model antibody, and related mass differences were significantly confirmed on the deglycosylated level. The CZE-CZE-MS setup is expected to be applicable to a variety of antibodies and thus, bares the potential to become a compelling tool for the detailed characterization of mAb charge variants.

1. Introduction

Monoclonal antibodies (mAbs) are considered to be the fastest growing group of pharmaceuticals, achieving sales of almost $75 billion in 2013 [1]. Starting with the first therapeutic mAb in 1986, nowadays more than 30 mAbs are available and currently approved by the US Food and Drug Administration [2]. The fields of application of mAbs comprise ,among others , the treatment of cancer, transplantations and cardiovascular, infectious or conic inflammatory diseases [3]. During manufacturing, purification and storage of these complex proteins, different variants besides the main form can occur [4]. Changes in micro-heterogeneity may lead to attenuated therapeutic efficacy [5]. Modifications encompass i.a. disulfide bond scrambling, change in glycosylation pattern, deamidation, pyroglutamate formation, loss of C-terminal lysine, oxidation (e.g. methionine) and amino acid substitution [6]. The characterization of such variants plays an important role regarding product quality, including technical development, release testing and control of manufacturing processes.

Important techniques for the characterization of mAbs comprise chromatographic approaches such as reversed-phase liquid chromatography (RPLC) [7,8], ion exchange chromatography (IEX) [9], size-exclusion chromatography [10], hydrophobic interaction chromatography (HIC) [11] and hydrophilic interaction liquid chromatography [12]. In addition, electrophoretic based separation techniques are commonly applied for the analysis of biopharmaceutical proteins, including capillary isoelectric focusing (CIEF) [13], capillary gel electrophoresis (CGE) [14] and capillary zone electrophoresis (CZE) [15–17,5,18]. A detailed comparison discussing benefits and limitations of these techniques can be found in recent review articles [19,1,2]. Utilizing chromatography, IEX mode, especially cation-exchange, is considered the benchmark for the determination of charge variants of therapeutic antibodies [9].

Due to their high selectivity and resolving power, miniaturized setup, low solvent and sample consumption, electrophoretic driven separation approaches have been commonly applied for

the analysis of biopharmaceuticals, especially on protein level [1]. CIEF [13] and CGE [14] are more quantitative and less time consuming alternatives to classical slab gel analysis. CZE represents the most straightforward electrophoretic separation technique, where ionic analyte species are separated based on their charge-to-size ratio. Thus, this technique is well suited for the separation of charge variants of intact antibodies [20]. Due to the small deviation in molecular mass between mAb charge variants (e.g. deamidation $\Delta m \approx 1$ Da), the difference in charge and not in hydrodynamic radius is often the important factor regarding separation efficiency. In order to increase resolution and avoid protein adsorption on the capillary wall, sophisticated electrolyte systems and capillary coatings have been developed [15,16,4,21,18]. During the last years, several CZE methods based on ε-aminocaproic acid (EACA) as background electrolyte (BGE) have been introduced [22–24,16,5]. Moreover, a generic EACA system [24], which was successfully applied for the charge heterogeneity profiling of 23 mAbs (pI = 7.4 to 9.2), has been tested in an extensive inter-laboratory study for the analysis of a monoclonal IgG1 showing impressive precision (standard deviations ~1 % for the main peak) [5].

Mass spectrometry (MS) has been demonstrated to be a powerful tool for the characterization of mAb variants [25]. Thus, MS is well established in the field of mAb analysis, providing information on the primary sequence, post-translational modifications and higher order structures [26]. In general, high resolution MS enables the discrimination of analyte species even with minor mass differences. Nevertheless, the isotopic envelope of high mass molecules such as mAbs is broad (~25 Da at full width at half maximum (FWHM)) limiting the provided information [2]. Due to overlapping small mass shifts caused by deamidation (+1 Da) or even larger shifts, e.g. oxidation (+16 Da) or N-terminal pyroglutamate formation (−18 Da), are difficult to resolve by MS alone. Therefore, electrophoretic or chromatographic separation is often performed prior MS detection. However, separation buffers or background electrolytes that are required for chromatographic and electrophoretic separations often interfere with the

electrospray ionization process. The aforementioned EACA based CZE system [24] represents a good example for considerable electrospray ionization (ESI) interference. Different approaches have been pursued in order to enable online CE-MS detection [27]. One way is to alter the electrolyte conditions (e.g. lower concentrations) and capillary coatings or to replace the non-volatile components by MS compatible electrolytes. However, it is challenging to maintain the resolution and selectivity of the original ESI-interfering BGE systems. Nevertheless, impressive results have been published recently by Ramsey and coworkers utilizing a microfluidic device for direct CZE-MS [4,21]. Another promising approach comprises the removal of the ESI-interfering components prior to MS detection. In this way, the existing, optimized and validated BGE systems for mAb characterization could potentially be preserved. In this regard, one straightforward concept represents the collection of previously separated fractions of mAb variants and either re-inject the fractions in a 2nd MS compatible separation dimension or pretreat the sample in between to remove interfering components prior to MS detection. Nevertheless, the collection of the low effluent in capillary electrophoresis (CE) is associated with high dilution of the desired analytes [28]. A more elegant approach is to hyphenate the ESI-interfering buffer system online with a 2nd separation dimension coupled to MS. Due to the low effluents and dimensions of CE in general, the application of another electrophoretic based separation technique as 2nd dimension is beneficial, especially CZE due to the available volatile BGE systems. In order to realize the online hyphenation of two electromigrative separation techniques, special coupling techniques are required due to the small volumes and strong electrical fields in CE. Over the last years, different types of sophisticated interfaces have been developed [29,28]. Recently, we have introduced a mechanical 4-port-valve with sufficient small volume (low nL range) for CE-CE-MS [30]. Different applications have been published, demonstrating the potential of this cutting-edge 2D setup [31,30,32].

Here, a heart-cut CZE-CZE-MS method was developed utilizing the aforementioned mechanical 4-port-valve for the MS characterization of mAb charge variants previously separated in a highly ESI-interfering generic EACA based electrolyte system. In this way, an assignment of mass spectrometric data to the respective peaks of the UV separation should be achieved. Glycosylated and deglycosylated samples of Trastuzumab were analyzed acting as a model mAb system. A high mass accuracy of the obtained MS spectra was aspired using constant deconvolution parameters and an external calibration of the MS data in near proximity to the mAb signal.

2. Materials and methods

2.1 Materials

Ultrapure water (electrical resistivity > 18 MΩcm) from an UltraClear UV system (Siemens water technologies, Günzburg, Germany) was used to prepare all BGEs, samples and rinsing solutions. Methanol (MeOH) (MS-grade), isopropanol (IPA) (MS-grade), sodium hydroxide (NaOH) (≥ 98%), ammonia solution (ROTIPURAN® 30% p.a.), ammonium hydrogen carbonate (≥ 99%, p.a.), formic acid (FAc) (ROTIPURAN® ≥ 98%, p.a., ACS) and acetic acid (HAc) (ROTIPURAN® 100%, p.a.) were obtained from Carl Roth (Karlsruhe, Germany). (Hydroxypropyl)methyl cellulose (HPMC) (M_w ≈ 26 kDa), triethylenetetramine (TETA) (≥ 97.0%), glutaraldehyde solution (50% in H_2O, suitable for photographic applications), poly(vinyl alcohol) (PVA) (M_w = 89 – 98 kDa, 99+% hydrolyzed), N-glycosidase F (rec. E.coli) and caffeine (99%) were purchased from Sigma Aldrich (Steinheim, Germany). Hydrochloric acid (HCl) (37% p.a.) and phosphoric acid (85% p.a.) were received from Häffner GmbH & Co KG (Asberg, Germany). ε-aminocaproic acid (pure) was obtained from Merck (Darmstadt, Germany). Trastuzumab (Herceptin® from F. Hoffmann-La Roche AG) was used. ESI-L Low

Concentration Tuning Mix (TM) was obtained from Agilent Technologies (Santa Clara, CA, USA). Bare fused silica (FS) capillaries (50 µm inner diameter (ID), 363 µm outer diameter) were purchased from Polymicro Technologies (Phoenix, AZ, USA). Nanosep 10K omega centrifugal devices were received from Pall Life Sciences (Ann Arbor, MI, USA). The C4N-4354-.02D microinjector 4-port-valve, including rotors (internal sample loops: 20 and 10 nL) and stators out of plastic material (Valcon E®, polytetrafluorethylene / polyether ether ketone composition), was obtained from VICI AG International (Schenkon, Swiss). The external UV detector applied in the 1st dimension of the CZE-CZE-MS setup was purchased from J+M Analytik AG (Essingen, Germany).

Neutral coating of capillaries with PVA, to prevent protein adsorption at the capillary wall and eliminating the electroosmotic flow, was performed in-house. A detailed description of the applied coating procedure can be found elsewhere [32]. Deglycosylation of Trastuzumab samples was performed with N-glycosidase F. 120 µL H_2O and 30 µL Trastuzumab (25 mg/mL) were added to a nanosep 10K omega centrifuge device. The formulation buffer was exchanged by 20 mM ammonium hydrogen carbonate (pH = 8, adjusted with 30% ammonia) at 6000 rpm, leading to a final volume of 40 µL and a theoretical concentration of 18.75 mg/mL Trastuzumab. Subsequently, samples were transferred to a tube containing 20 units of N-glycosidase F followed by incubation (37°C, 24 h) to achieve complete deglycosylation. In order to deglycosylate higher concentrated mAb samples, the amount of N-glycosidase F was adapted accordingly. Samples were analyzed directly without further purification.

2.2 CZE-UV

CZE-UV measurements were performed on an HP ^{3D}CE electrophoresis instrument from Agilent (Agilent Technologies, Waldbronn, Germany) using capillaries with an ID of 50 µm and a length of 60.0 cm. Bare fused silica capillaries were preconditioned every morning by

rinsing (approximately 1 bar) with 0.1 M HCl (10 min) and EACA based BGE (10 min) followed by the application of 30 kV for 10 min. The BGE consisted of 380 mM EACA, 1.9 mM TETA and 0.05% w/w HPMC (pH = 5.7, adjusted with HAc), adapted from He *et al.* [24]. Prior to each run, the capillary was rinsed (approximately 1 bar) consecutively with 0.1 M HCl (5 min), H_2O (2 min) and BGE (4 min). For long term or overnight storage the capillary was rinsed (approximately 1 bar) with 0.1 M phosphoric acid for 5 min and placed in vials filled with H_2O. Samples were injected hydrodynamically (50 mbar, 24 s). Constant voltages of 30 or 10 kV were applied during separations and UV detection was performed at 214 nm wavelength.

2.3 CZE-ESI-MS

CZE-ESI-MS was performed on an HP ^{3D}CE electrophoresis instrument from Agilent coupled to a *compact* quadrupole – quadrupole time of flight (QqTOF) instrument from Bruker Daltonik (Bremen, Germany). In-house PVA coated capillaries with a total length of 60.0 cm were applied. The CZE-ESI-MS coupling was performed with a commercial triple-tube sheath liquid interface (Agilent Technologies, Waldbronn, Germany). PVA capillaries were positioned in such a way that approximately 0.05 mm stuck visible outside of the sheath liquid sprayer needle. Different BGE solutions were applied: 0.2 and 1 M FAc; 0.5, 1, 2, 3 and 5 M HAc. The sheath liquid (SL) was based on IPA:H_2O (1:1) and different amounts (0.2 and 1.0% (v/v)) of FAc and HAc were tested as additives. The SL was delivered at a flow rate of 4 µL/min by a syringe pump (Cole-Parmer®, IL, USA) equipped with a 5-mL syringe (5MDF-LL-GT, SGE Analytical Science, Melbourne, Australia). 10 kV were applied for separation. Samples were injected hydrodynamically (50 mbar, 24 s). Two different MS methods were applied: A wide range method for the simultaneous detection of EACA clusters and antibodies and a high mass method optimized for sensitive mAb detection. The most important parameters are summarized in

Table 1. The source voltages were set to 0 V for a short period of time (0.1 min) at the beginning of both methods in order to avoid contamination of the instrument between acquisitions. Data processing was carried out by Bruker Compass Data Analysis software (Version 4.2, Bruker Daltonik, Bremen, Germany). For overnight or long-term storage, the PVA capillaries were flushed (approximately 1 bar) with H_2O and dried with air (empty vial) for 10 min, respectively.

2.4 Heart-cut CZE-CZE-MS

The EACA based CZE-UV method was online hyphenated to CZE-MS by implementing a mechanical 4-port-valve as interface. Both electrophoretic dimensions and the 4-port-valve interface are described individually in the following sections:

2.4.1 First dimension: CZE-UV

The CZE-UV measurements in the 1st dimension were performed on an HP ^{3D}CE electrophoresis instrument from Agilent (Agilent Technologies, Waldbronn, Germany). A 40.0 cm long FS capillary was connected through an external UV detector to the 4-port-valve at junction "S" as displayed in **Figure 1**. The UV detector was located 4.5 cm in front of the valve. A 20.0 cm long FS capillary was mounted to the valve at junction "W". The other end of the capillary was placed in an external outlet vial. A grounded ampere meter was connected to this vial acting as counter electrode and to monitor the current stability. The same BGE, preconditioning (10 instead of 30 kV) and overnight procedures were used as applied in the one-dimensional approach described above. Samples were injected hydrodynamically (50 mbar, 24 s). 10 kV were applied for separation and 214 nm was selected as detection wavelength. Separation was performed till the desired analytes reached the UV detector. At this point, the separation was stopped and the analytes were transferred into the loop of the valve

by applying 50 mbar. Flow rates were determined prior to each analysis by injecting a small caffeine plug (1 mg/mL, 50 mbar, 12 sec) and flushing with 50 mbar till it passed the external UV detector. Considering the required time of the caffeine plug to pass the UV detector and the capillary geometries the flow rates (50 mbar) were calculated as described in detail in our previous work [32].

2.3.2 4-port-valve

A mechanical 4-port-valve was applied as interface between the 1st and 2nd dimension which consisted of three major parts (see **Figure 1A**): the stator, the rotor with sample loop and the motor. All three parts were pushed tight together via 2 screws to avoid leakages during the measurements. A more detailed description of the valve and its characteristics can be found elsewhere [31,30]. Two different sample loop sizes (20 and 10 nL) were applied during the experiments (see **Figure 1C**). If not particular stated otherwise, the rotor with the 10 nL sample loop was used. The capillaries of the 1st and 2nd dimension were connected to the stator with 4 junctions (S, W, P and C) using finger-tight screws. During the 1st dimension the mechanical valve was kept in "Loading Position" (see **Figure 1B**, upper scheme). When the desired analytes were located in the sample loop the valve was switched to "Inject Position" (see **Figure 1B**, bottom scheme). After the CZE-ESI-MS analysis, the valve was switched back to "Loading Position".

2.3.3 Second dimension: CZE-ESI-MS

CZE-ESI-MS was performed with an HP ^{3D}CE electrophoresis instrument from Agilent (Agilent Technologies, Waldbronn, Germany) using in-house PVA coated capillaries (total

length 80.0 cm). The capillary was cut into two parts (40.0 cm, respectively) and connected to the valve at junction "C" and "P" (see **Figure 1**). The BGE was composed of 2 M HAc. During the separation in the 1st dimension the PVA capillaries were conditioned (approximately 3 bar, 10 min) with BGE. After the analytes were transferred to the 2nd dimension, 10 kV were applied for separation. For overnight or long-term storage, the PVA capillaries were flushed (approximately 1 bar) with H_2O and dried with air (empty vial) for 10 min, respectively.

The same sheath liquid interface and MS (including software) was applied as for the one-dimensional CZE-ESI-MS experiments. The SL (IPA:H_2O (1:1) + 1% HAc) was delivered at a flow rate of 4 µl/min. If not stated particularly otherwise, the aforementioned "high mass" method parameters were selected for two-dimensional experiments (see **Table 1**). Directly after the analyte reached the MS detector, a 20 µl plug of calibrant (TM solution) was injected via the SL connection utilizing the integrated 6-port-valve of the MS instrument. The tuning mix signals were used to perform an external calibration of each individual data file. A maximum entropy deconvolution was performed with the 12 most abundant charge states (2680 – 3400 *m*/*z*) of the raw mass spectrum.

3. Results and discussion

3.1 Method development

Prior to the application of the complete CZE-CZE-MS setup, the separation dimensions were developed individually in a one-dimensional approach. For the 1st dimension, a generic CZE-UV method for mAb charge variant separation was selected using typical values for an EACA based electrolyte system [5]. The maximum applicable high voltage utilizing the mechanical 4-port-valve is limited to ~15 kV, due to material properties and geometries of the valve as described in detail in our previous work [31,30]. For this reason, the separation profile of

Trastuzumab (12.5 mg/mL) applying 30 kV and 10 kV were compared regarding peak number and resolution using one-dimensional CZE-UV (data not shown). Although the analysis time increased accordingly, the observed peak number remained the same and the general pattern was preserved. Furthermore, a one-dimensional separation of Trastuzumab through the 4-port-valve using the complete 2D setup was performed, as displayed in **Figure 2**. No noticeable decline in profile quality was observed by implementing the 4-port valve. Moreover, the achieved separation pattern of Trastuzumab is in good agreement with the observations described in the literature [22], showing a small basic peak in the front of the main variant and some acidic forms migrating subsequently.

The main purpose of the 2nd CZE dimension is to separate the remaining ESI-interfering molecules, including the highly concentrated EACA, originating from the BGE of the 1st dimension from the desired mAb variant. For this reason, Trastuzumab (12.5 mg/mL) was dissolved in the BGE of the 1st dimension and analyzed via one-dimensional CZE-MS. For this experiment the settings of the QqTOF-MS were adapted in a way that both, EACA clusters and the intact mAb molecules, could be detected which is also referred as “wide range” MS method (see **Table 1**). A variety of different volatile BGE compositions were tested: 0.5 and 1 M FAc; 0.5, 1, 2, 3 and 5 M HAc (data not shown). No separation of mAb and EACA could be achieved utilizing FAc based electrolytes. Using 1 M HAc as BGE the mAb begins to get separated from the broad EACA cluster signals which migrate in front of the mAb. Increasing the BGE concentration to 2 M HAc the separation was improved and an example electropherogram is displayed in **Figure 3**. Higher concentrations of HAc did not result in any additional improvement regarding separation efficiency. Thus, 2 M HAc was selected as BGE for all further experiments. The very broad signal of the EACA clusters migrating directly in front of the mAb peak suggests the presence of effects similar to transient isotachophoresis, leading to a sharp and concentrated mAb signal. Subsequently, the same sample was analyzed with an

injection via the 4-port-valve using the complete 2D setup in order to ensure that the separation efficiency of the one-dimensional CZE-ESI-MS is preserved. Therefore, the capillary of the 1st dimension was filled completely with sample prior to switching the valve for injection in the 2nd dimension. In this way, analogous results were achieved. In order to improve the sensitivity of the MS measurement, the influence of the SL composition on signal intensity of the mAb signal was evaluated. First, the MS setting were adapted to achieve the highest possible intensity for high mass molecules using TM infusion (see **Table 1**). Subsequently, the influence of acidic additives in the SL was tested via one-dimensional CZE-ESI-MS analyzing the sample solved in BGE. The SL was composed of IPA/H2O 1:1 and different amounts of FAc and HAc were added: 0.2 and 0.5% FAc; 0.2, 0.5 and 1% HAc (data not shown). In general, HAc delivered higher mAb signals compared to FAc. The highest sensitivity was achieved with 1% HAc and consequently, this SL composition was selected for all further experiments.

One crucial part of the 2D setup represents the transfer of analytes from the 1st to the 2nd dimension. Therefore, a precise positioning of desired analyte peaks inside the sample loop is mandatory. An obvious and simple approach is to calculate the required time for the analyte to migrate from the external UV detector to the center of the sample loop during the separation in the 1st dimension. In principle, only the time for the analyte to reach the external UV detector and the respective distances (capillary inlet → external UV and external UV → center of loop) are required. Still, this methodology premises a constant migration velocity throughout the whole separation in the 1st dimension. However, due to i.a. field inhomogeneities directly after injection and possible dead volumes caused by the capillary junctions of the valve, the migration velocity of the analytes appears to be not perfectly consistent throughout the separation. In this way, a precise cutting using this method is challenging. A second, more promising, methodology is to stop the analysis when the desired analyte reaches the external UV detector and push the analyte into the sample loop applying defined low pressure (50 mbar).

The required time was calculated using the flow rate at 50 mbar which was determined prior to the analysis. A detailed description of this procedure can be found in our previous work [32]. Briefly, a small plug of caffeine (1 mg/mL) was injected (50 mbar, 12 sec) and pushed with 50 mbar towards the valve till it passed the external UV detector. The required time was used to calculate the flow rate. In order to compensate even minor variations in the flow profile, potentially resulting from switching the valve, and to achieve a very precise cutting, the flow determination was performed prior to each individual analysis.

The obtainable mass accuracy and precision of the QqTOF-MS instrument plays an essential role for the characterization of mAb variants, especially due to their high molecular mass and structural similarities. In order to achieve highly accurate results an external calibration of the MS data in a close time frame to the analyte signal was implemented in the 2nd dimension, which is exemplary displayed for a cut of the main variant of Trastuzumab (18 mg/mL) in **Figure 4a**. Thereby, a TM solution is infused via the SL tubing directly after the mAb is detected in the MS using the integrated 6-port valve of the QqTOF-MS instrument. The resulting tuning mix signals were used to recalibrate the MS spectra during data evaluation (quadratic regression model). In this way, variations in the instrument performance could be corrected subsequently.

3.2 Glycosylated mAb samples

The mass spectrum of the cut main variant of glycosylated Trastuzumab (18 mg/mL), demonstrating the general applicability of the 2D setup, is displayed in **Figure 4b**. Charge states ranging from +38 to +62 were observed in the raw mass spectrum. The CZE-CZE-MS setup was validated regarding migration time, single intensity, current and flow rate stability by cutting the main variant of Trastuzumab several times (12.5 mg/mL). The results of the

validation are summarized in **Table 2**. The current in both dimensions was stable (relative standard deviation $(RSD)_{intra}$ = 1.2 – 1.8%; RSD_{inter} = 1.5 – 2.9%) and no current leakage was observed throughout all measurements. As stated before, the flow rate in the 1st dimension was determined prior to each run in order to ensure the highest possible precision of the cutting procedure. Nevertheless, RSD values of 0.9 (intra-day) and 4.4% (inter-day) were achieved for the flow rate. The intra-day precision of the migration time in the 1st and 2nd dimension were 0.8 and 1.7%, respectively. The inter-day precision of the migration time in the 1st and 2nd dimension were determined to be 3.1 and 6.2%, respectively. The external UV signal did only show minor variation in intensity (RSD_{intra} = 1.1%; RSD_{inter} = 2.1%). The intra- and inter-day RSD values of the MS signal intensity in the 2nd dimension were 12.1 and 16.9%, respectively. These values are in the expected range for ESI-MS measurements without the application of specific internal standards. Since the main purpose of the CZE-CZE-MS setup was the characterization and not quantification of mAb variants, these RSD values were considered acceptable for this kind of application.

The raw as well as the deconvoluted mass spectra showed the typical glycoform pattern of Trastuzumab as illustrated in **Figure 4b** and **c**. The mass differences (161.5 – 162.1 Da) between the three main glycoforms match well with the expected difference of one hexose (162.1 Da). For the three highest abundant glycoforms G0F/G0F, G0F/G1F and G1F/G1F (G0F/G2F) an average mass of 148059.1 ± 0.8, 148220.1 ± 0.5 and 148381.6 ± 0.6 (n = 5) was determined, respectively. This corresponds to a deviation of +20.2, +13.0 and +8.4 ppm compared to the theoretically calculated average masses (148056.1, 148218.2 and 148380.4 Da) using the "representative isotopic distributions" of IUPAC [33]. The obtained masses are well within expected accuracy, taking the mass spectrometric data of other published works for Trastuzumab in consideration (~+22 to +38 ppm, calculated based on illustrated mass spectra and data) [25,2]. The presented as well as the literature data show a general tendency to slightly

higher masses compared to the calculated values. There are several possible explanations for this observation, including a not considered post translational modification (PTM), a shift caused by the mass calibration procedure, the formation of adducts affecting the average mass or a divergent isotopic distribution of elements. Trastuzumab has been studied thoroughly over the years on all types of analytical levels. Thus, the presence of an unknown, not considered, PTM causing a minor positive shift in mass can be excluded. In addition, different calibration models (quadratic and linear) were tested. The applied calibration model did only cause a minor, non-systematic difference in the resulting mass (e.g. G0F/G1F: quadratic 148220.1 ± 0.5 Da; linear 148220.2 ± 0.5 Da). Therefore, the applied calibration model can be excluded as source for the observed slightly higher masses. Furthermore, the influence of adduct formation on the determined mass was investigated. Adducts, including ammonium or sodium, are not fully resolved from the protonated species due to the broad isotopic pattern of the intact antibody (FWHM ≈ 25 Da). It does not appear unlikely for one or a few of the protons (38 – 62) to be exchanged by another cation. For this purpose, a simple simulation has been performed, taking the mass distribution of the protonated form, the sodium adduct and the ammonium adduct of the mAb into consideration. If in 20 % of cases one proton is exchanged by one ammonium (or in 41 % by one sodium), a shift of +1 Da is already observed for the mAb signal. This corresponds to a proton exchange of 0.3 – 0.5% by ammonium (or 0.7 – 1.1% by sodium) considering all available protons. On the other hand, the relative amount of ^{13}C in the "representative isotopic distributions" of IUPAC ($\delta^{13}C$ = -37) [33] is already out of range regarding the natural isotopic distribution of carbon in organic material (C3, C4 and CAM plants: $\delta^{13}C$ = -10 to -33) [34]. Thus, a deviation in the isotopic distribution of carbon could be a plausible explanation for the observed masses. Taking the entire width of the natural organic carbon distribution into account, the theoretical calculated values for the three main glycoforms are 148057.2 (G0F/G0F), 148219.4 (G0F/G1F) and 148381.5 (G1F/G1F and G0F/G2F) with a range of ± 0.8, respectively. With these values a mass deviation between +0.6 to +12.6 ppm

was determined for the measured masses. By taking the measurement uncertainty (2σ) in consideration, the observed masses are in agreement with these calculated masses. In conclusion, an accurate assessment for the mass accuracy was not possible, due to missing information regarding the exact isotopic distribution of elements, especially carbon, in Trastuzumab. Additional information, e.g. the origin of the amino acids applied in the manufacturing process of Trastuzumab, could result in a more precise values for the theoretical calculated masses, highlighting the challenge of accurate mass calculation for high mass molecular species.

In general, basic and acidic variants of mAbs are considerably less concentrated compared to the main form. Thus, it is important to verify that the resulting measured mass is not significantly influenced by the concentration. In this regard, the main variant was cut 5 times on 3 different concentration levels: 30, 18 and 9 mg/mL. No significant difference was observed between all three concentration levels (G0F/G1F: 148220.0 ± 0.4, 148220.1 ± 0.5 and 148220.4 ± 0.5 Da, respectively).

Subsequently, the most intensive acidic variant of Trastuzumab (30 mg/mL) was cut. A slightly higher mass (~2-3 Da) was observed compared to the main variant (data not shown). The signal was very noisy and thus, not sufficient for a repeatable accurate mass determination (standard deviation 1.8 Da; n = 5). Therefore, a detailed interpretation of the data was not possible. Furthermore, the analysis of higher concentrations did not result in better data quality, due to loss of resolution and peak broadening in the 1st dimension. Nevertheless, a slightly higher mass of the intact mAb in combination with a noticeable shift to higher migration times implies the presence of potential deamidation processes.

3.3 Deglycosylated mAb samples

In order to increase the sensitivity and simultaneously simplify the spectrum, a deglycosylation of Trastuzumab samples was performed using N-glycosidase F. An electropherogram of the 1st dimension analyzing deglycosylated Trastuzumab (6 mg/mL) is shown in **Figure 5a**. It is apparent that the mAb profile changed compared to the glycosylated sample (see **Figure 2**), resulting in distinct higher intensities for the acidic variants. This fact implies a chemical alteration of the sample induced by the N-glycosidase F treatment. First, the main variant was cut 5 consecutive times. An example deconvoluted mass spectrum of the main variant is displayed in **Figure 5b**. A mass of 145171.5 ± 0.4 Da was observed. This corresponds to a deviation of +20.3 ppm compared to the theoretical calculated mass (145168.6 Da) based on the average carbon distribution in plants. No signals of remaining glycosylated variants were observed. Similar results were obtained analyzing a less concentrated Trastuzumab sample (4 mg/mL), delivering a mass of 145171.5 ± 0.5 Da (n = 4) for the main mAb variant. Thus, for both, glycosylated and deglycosylated samples, mass spectra with high reproducibility (standard deviation < 1 Da) and mass accuracy (lower ppm range) at different concentration levels have been obtained.

Subsequently, the highest abundant acidic variant A2 was cut multiple times (n = 5). An example of a deconvoluted mass spectrum can be found in **Figure 5c**. As already indicated by the analysis of the glycosylated sample, a slightly higher mass compared to the main variant was observed (145173.5 ± 0.6 Da), which corresponds to a shift of +2.0 Da. The mass difference to the main variant was confirmed to be statistically significant (t-test: $\alpha = 0.05$; $p < 10^{-5}$). Furthermore, the acidic variant A3 was cut (n = 5). In order to achieve sufficient signal intensity a higher concentrated deglycosylated sample (30 mg/mL) was analyzed. A mass of 145174.6 ± 0.8 Da was detected, which corresponds to a mass difference of +3.1 Da compared to the main variant. The mass difference (Δm = 1.1 Da) between A2 and A3 was confirmed to be statistically significant (t-test: $\alpha = 0.05$; $p = 0.018$). As stated before, these acidic variants can

most likely be attributed to deamidation products, due to the slightly higher masses combined with a considerable shift to higher migration times. Thus, the acidic variant A2 and A3 could correspond to a double and triple deamidation, respectively. In principle, Trastuzumab possesses several positions in the peptide chain that can be potentially deamidated [35]. Most studies are focused on the Asn30 in the light chain. However, Asn55 located in the CDR2 region of the heavy chain is also known to be susceptible to deamidation at elevated pH values (> 7.5). This hypothesis is additionally substantiated by the elevated intensity of the acidic variants after deglycosylation at pH = 8 (see **Figure 2** and **5a**). Furthermore, N-glycosidase F treatment is known to cause significant amounts of additional chemical deamidation [36]. Summarized, the occurrence of deamidation products represents a plausible and compelling explanation for the identity of the acidic variants A2 and A3.

4. Conclusion

MAb charge variants are characterized routinely in biopharmaceutical industry applying EACA based CZE-UV methods [5]. Utilizing such an approach implementing a generic EACA electrolyte, we developed and validated a heart-cut CZE-CZE-MS method for the detailed mass spectrometric characterization of mAb charge variants. In this way, a clear online assignment of mass spectrometric data to the peaks of this ESI-interfering CZE-UV system was achieved online for the first time. Highly precise mass data (standard deviation 0.4 – 0.8 Da) of glycosylated and deglycosylated variants of Trastuzumab, acting as model mAb system, were obtained. The measured and calculated masses are in good agreement (lower ppm range). Remaining uncertainty originates *i.a.* potential adduct formation during the measurement and relative isotopic abundances used for the calculation, highlighting the challenge of accurate mass determination for high mass molecular species.

Combining the electrophoretic separation and the highly precise masses, potential deamidation products were identified for an intact antibody. The entire system is expected to be applicable to a variety of different BGE conditions (e.g. EACA concentration). Thus, extensively optimized EACA methods for specific mAbs can be coupled to mass spectrometry utilizing CZE-CZE-MS. Still, there is room for improvement regarding the sensitivity and sample pretreatment (generation of additional deamidation caused by deglycosylation procedure) to characterize even very low abundant charge variants. The presented CZE-CZE-MS system bares the potential to become a compelling tool for the characterization of mAb charge variants.

Acknowledgements

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Figure Captions

Figure 1: General setup of the applied CZE-CZE-MS system. Scheme of the three major parts of the mechanical 4-port-valve **(A)**: motor (I), rotor including the sample loop (II) and the stator with associated capillary connections (III). Scheme of the complete 2D setup **(B)**. The inlet (CE1) and outlet capillary, which is placed in a grounded external vial (EV), of the 1st dimension are connected to channel S and W of the 4-port-valve, respectively. An external UV detector cell is positioned 4.5 cm in front of the valve through the inlet of the 1st dimension. The inlet (CE2) and outlet capillary (MS) of the 2nd dimension are connected to channel P and C of the 4-port-valve, respectively. During the separation of the 1st dimension the mechanical valve is kept in loading position (upper part) where the sample loop is connected to channel S and W. When the desired analyte is located in the sample loop, the valve is switched to inject position (lower part) transferring the analyte from the 1st to the 2nd dimension. Subsequently, 10 kV are applied for separation. Scheme of applied rotors **(C)**: 10 and 20 nL sample loop volumes.

Figure 2: Electropherogram of Trastuzumab (12.5 mg/mL) in the 1st dimension: CZE-UV separation was performed through the 4-port-valve with the complete 2D setup and a total capillary length of 60 cm (UV located at 35.5 cm from the injection end). The BGE contained 380 mM EACA, 1.9 mM TETA and 0.05% HPMC (pH = 5.7). UV detection was performed at 214 nm and 10kV were applied for separation. The mAb profile is divided into three regions: basic **(B)**, main **(M)** and acidic variants **(A)**.

Figure 3: Electropherogram of Trastuzumab (12.5 mg/mL) in the 2nd dimension: CZE-MS separation was performed in PVA capillaries (total length: 60 cm) and 2 M HAc as BGE. MS setting were adjusted to the “wide range” method parameters (see **Table 1**) and +10 kV were

applied for separation. Extracted ion electropherograms of the EACA dimer (green dashed line) and the sum of the 15 highest abundant mAb charge states (red line) are displayed. Sufficient separation of EACA clusters and mAb was achieved.

Figure 4: Base peak electropherogram (2500 – 5000 *m*/*z*) of the 2nd dimension cutting the main variant of Trastuzumab (18 mg/mL) via CZE-CZE-MS **(A)**. The electropherogram was divided into two sections: migration of mAb towards MS (I) and infusion of TM via SL tubing for post analysis mass calibration (II). The inset contains a scheme of the 6-port-valve integrated in the QqTOF-MS instrument. During section I the sample loop (20 µL) of the 6-port-valve was filled with TM (position waste) and infused to the MS via the SL tubing during section II (position source). The TM signals were used to recalibrate the MS data. Mass spectrum of main variant of Trastuzumab **(B)**. Charge states ranging from +38 to +62 were observed. Deconvoluted mass spectrum of Trastuzumab main variant **(C)**. The three highest peaks of the spectrum were assigned to the corresponding glycoform. The inset contains the structure of the three common glycans (G0F, G1F and G2F) present in Trastuzumab: fucose (red triangle), N-acetylgalactoseamin (blue square), mannose (green circle), galactose (yellow circle).

Figure 5: CZE-UV electropherogram of deglycosylated Trastuzumab (6 mg/mL) **(A)**. Separation was performed with the CZE-CZE-MS setup. 1st dimension: a separation voltage of +10 kV, 380 mM EACA, 1.9 mM TETA and 0.05% HPMC (pH = 5.7) as BGE, and bare fused silica capillaries (total length 60 cm) were used. 2nd dimension: a separation voltage of +10 kV, 2 M HAc as BGE, and PVA coated capillaries (total length 80 cm) were applied. Raw and deconvoluted mass spectra of main variant (10 nL cut) **(B)**, acidic variant A2 (20 nL cut) **(C)**

and acidic variant A3 (20 nL cut **(D)**. For the cut of A3 a higher concentrated sample was applied (50 mg/mL).

Figures

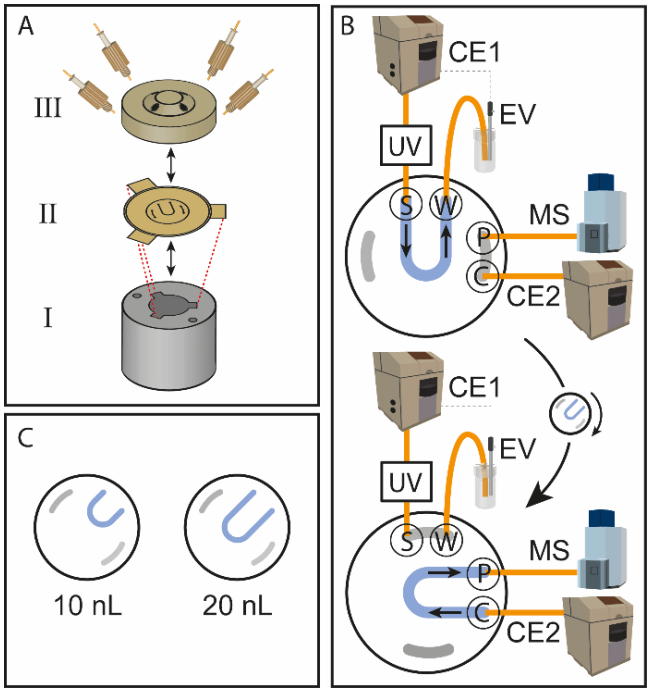
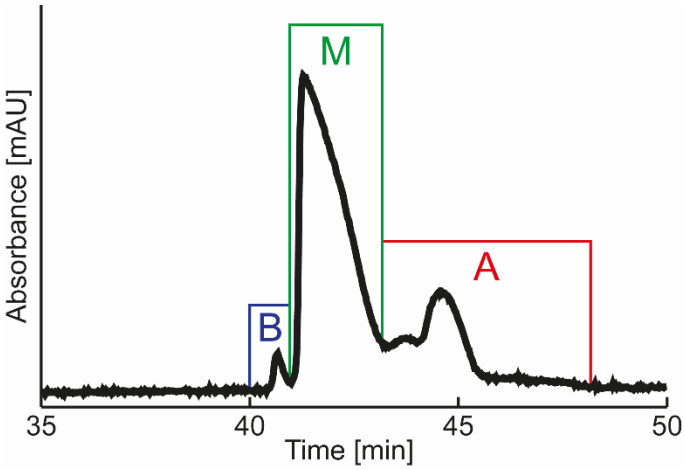


Figure 1



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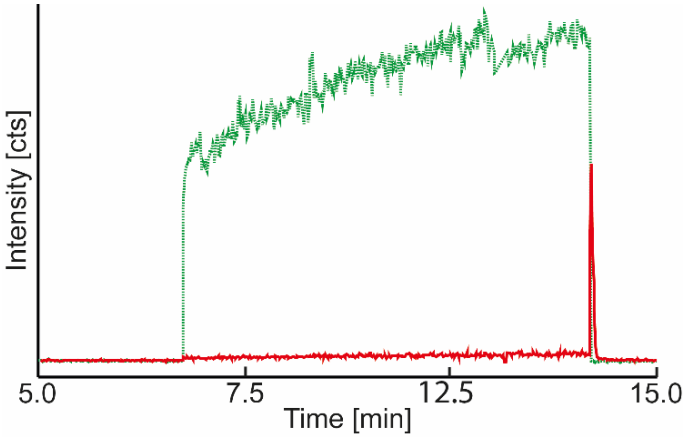
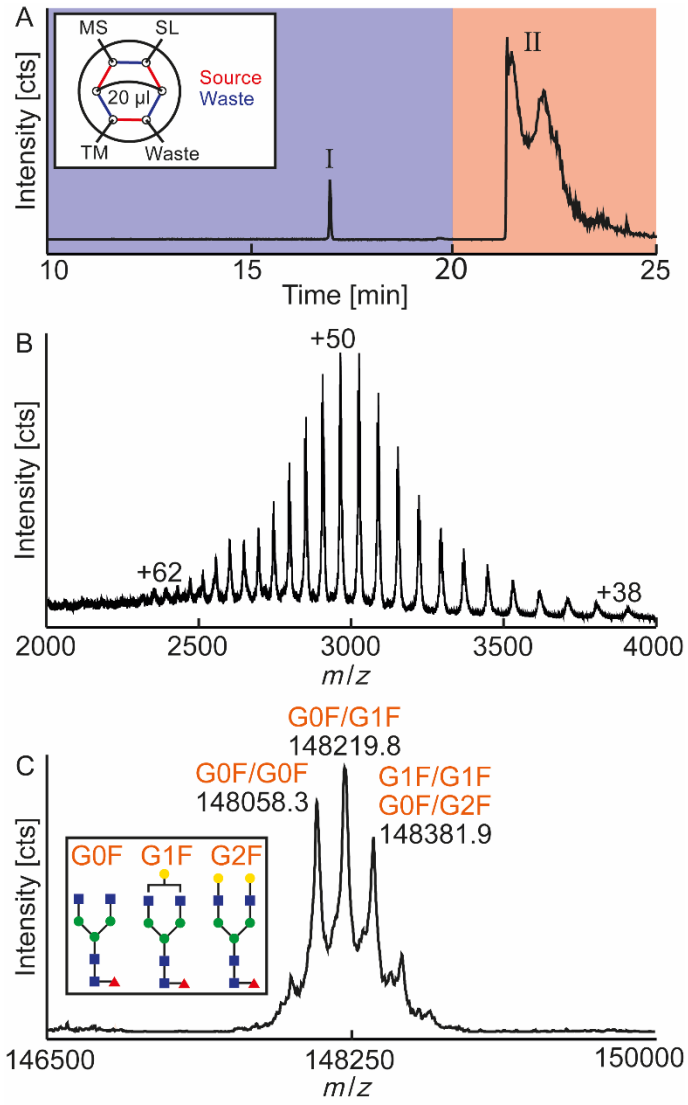


Figure 3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MS
SL
20 µl
Source
Waste
TM
Waste
Intensity [cts]
I
II
10
15
20
25
Time [min]
B
+50
Intensity [cts]
+62
+38
2000
2500
3000
3500
4000
m/z
C
G0F/G1F
148219.8
G0F/G0F
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G2F
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150000
m/z

Figure 4

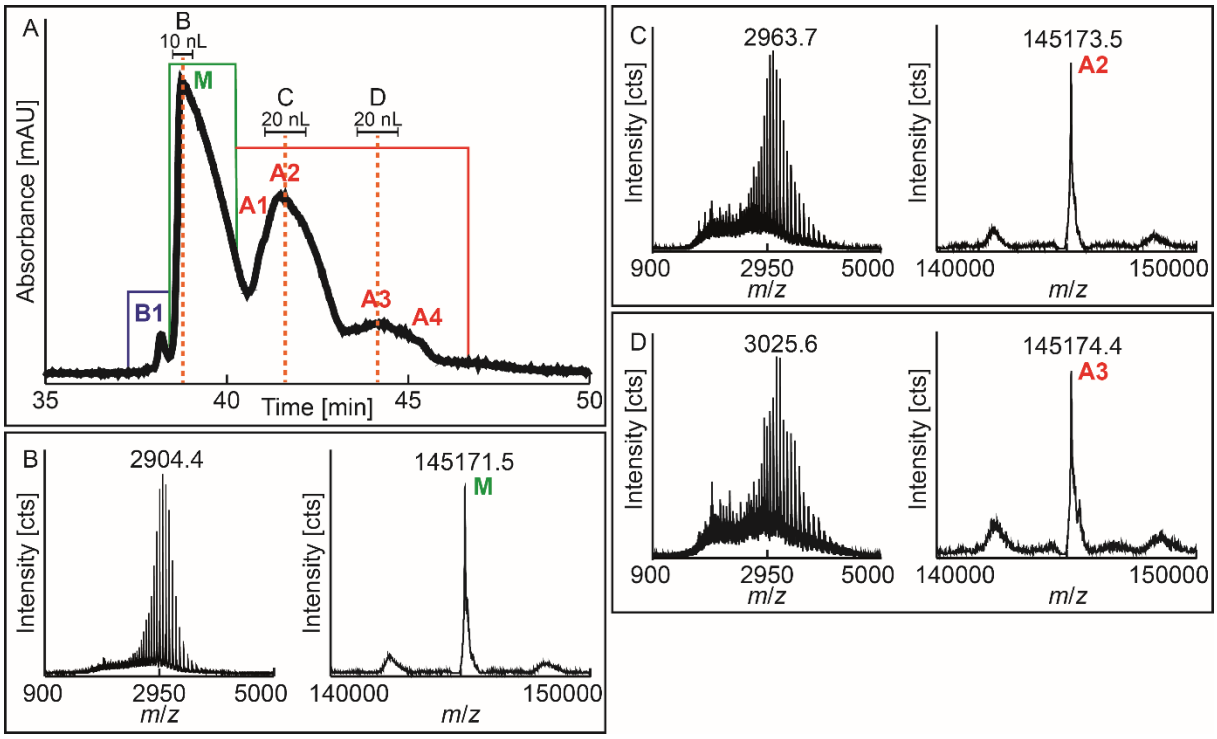
A
B
10 nL
M
C
20 nL
D
20 nL
A2
A1
A3
A4
B1
Absorbance [mAU]
35
40
Time [min]
45
50
B
2904.4
Intensity [cts]
900
2950
5000
m/z
145171.5
M
Intensity [cts]
140000
150000
m/z
C
2963.7
Intensity [cts]
900
2950
5000
m/z
145173.5
A2
Intensity [cts]
140000
150000
m/z
D
3025.6
Intensity [cts]
900
2950
5000
m/z
145174.4
A3
Intensity [cts]
140000
150000
m/z

Figure 5

Tables

Table 1: Parameters of the applied Q-TOF MS methods (wide range and high mass)

Parameter	Wide range	High mass
End plate offset	500 V	500 V
Capillary voltage	5000 V	5000 V
Nebulizer	0.5 bar	0.5 bar
Dry gas	3.0 L/min	3.0 L/min
Dry temperature	170°C	170°C
Mass range	100 – 5000 *m/z*	700 – 5000 *m/z*
Pre pulse storage	32.0 µs	32.0 µs
Collision cell transfer time	50 (50%) – 130 (50%) µs	140 µs
Collision cell RF	500 (50%) – 1900 (50%) Vpp	2100 Vpp
Collision cell	15 eV	30 eV
Quadrupole ion	7 eV	30 eV

Table 2: Results of the validation of the CZE-CZE-MS system

	Intra-day RSD [%] n = 5				Inter-day RSD [%] n = 10			
Dimension	**Current**	**Flow Rate**	**Migration Time**	**Peak Intensity**	**Current**	**Flow Rate**	**Migration Time**	**Peak Intensity**
1st	1.8	0.9	0.8	1.1	2.9	4.4	3.1	2.1
2nd	1.2	-	1.7	12.1	1.5	-	6.2	16.9

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Achievments

2015 **Metrohm best young scientist award for innovative science**, *Fast and sensitive protein analysis with online coupled capillary isoelectric focusing - capillary zone electrophoresis - mass spectrometry*, ITP 2015 Helsinki, FIN.

Experience

Current position

since 02/17 **Product manager for maintenance and workflow solutions**, *Agilent Technologies*, Waldbronn.

Education

09/14–01/17 **PhD student, research associate**, *at University of Tuebingen, Institut of Pharmaceutical Science (Prof. Laemmerhofer) as a research associate at Aalen University in the working group of Prof. Neusuess.*
Topic: Hyphenation of capillary based electro driven and chromatographic separation techniques

03/14–08/14 **MA Thesis**, *Institute for Science and Technology Austria, Vienna and Institut of Pharmaceutical Science, University of Tuebingen*.
Topic: Quantification of Riboflavin, FMN and FAD in cellular models with CE-LEDIF

03/13–08/14 **MA: Analytical and Bioanalytical Chemistry**, *Faculty of Chemistry, Aalen University*, Final grade: 1,1.

03/09–02/13 **BA: Chemistry (Focus: Analytic)**, *Faculty of Chemistry, Aalen University*.
Thesis in cooperation with Dr. Kade Pharma. Topic: Optimization and Validation of Karl-Fischer titration methods

03/11–08/11 **Internship semester**, *German Aerospace Center (DLR)*, Stuttgart.
Topic: Salts as heat transporter in solar thermal power plants

Working experience

03/09–08/14 **Chemical-technical assistant (Part time employment)**, *Quality control with HPLC at Dr. Kade Pharma*, Konstanz.
Optimization and performance of HPLC methods

01/08–02/09 **Chemical-technical assistant**, *Quality control with HPLC at Dr. Kade Pharma*, Konstanz.
Optimization and performance of HPLC methods, responsible for solvent management and sampling

08/06–12/07 **Chemical-technical assistant**, *Quality control with HPLC and GC at Cilag AG*, Schaffhausen, CH.
Performance of HPLC and GC methods

Given university courses (Aalen University)

WiSe 14/15	**Lab course**	*mass spectrometry*
SoSe 15	**Science project**	*HPLC-QqQ MS*
SoSe 15	**Science project**	*Fast CIEF of proteins*
SoSe 15	**Lecture**	*Separation science (HPLC and CE)*
SoSe 15	**Lab course**	*HPLC*
WiSe 15/16	**Science project**	*CIEF for 2D Applications*
SoSe 16	**Science project**	*CE and CIEF for monoclonal antibodies*

Software experience

Excellent ChemStation (HPLC/GC/CE), Microsoft Office, Adobe Suite (Photoshop, Illustrator, InDesign), LaTeX

Advanced Matlab, Data Analysis and qTOF Control (Bruker Daltonics)