Capillary Electrophoresis-Mass Spectrometry for the Identification of Aminopyrene Trisulfonic Acid Labeled Glycans

Dissertation

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Contents

Chapter 1

Introduction

1.1 Glycosylation of Biopharmaceutical Products

The application of proteins in the biopharmaceutical field is rapidly increasing. In the last decade one in four of all new drugs developed and placed on the market have been biopharmaceuticals. In general, biopharmaceuticals are therapeutics produced by modern biotechnology, respectively "produced by means other than direct extraction from a native (non-engineered) biological source" [1, 2]. Since the 1990s the term "biopharmaceutical" includes drugs based on proteins, nucleic acids and engineered stem and other therapeutic cell lines [3]. It is estimated that in the next decade approximately 30% of newly licensed drugs will be based on antibody products [4-7]. Protein based drugs, such as antibodies, undergo a variety of different posttranslational modifications (PTM) as proteolytic processing, disulfide bond formation, sulfatation, amidation, glycosylation and carboxylation. Thereof, glycosylation represents one of the most characteristic as well as most complex PTM of biopharmaceuticals. Studies have shown that more than 50% of all proteins are glycosylated [8, 9]. Monoclonal antibodies for instance are mainly based on immunoglobuline G (IgG) and therefore are mostly glycosylated.

Glycosylation has influence on the protein folding process, chemical and physical stability, the physiological life time as well as many biological processes on protein and /or cellular basis. Therefore, eludicating the glycosylation patterns of pharmaceutically employed glycoproteins is of high importance in terms of product quality and therapeutic efficacy [4, 10]. The same protein can be glycosylated differently, related to factors as enzyme activity, monomer availability or protein dwelling time in Golgi and endoplasmic reticulum. Hence, even the PTM of native molecules in their natural environment undergoes various modifications. In cultured cells the type and content of cultivation media, including pH and the availability of nutrients and precursor molecules may have influence on the glycosylation as well as diverse cell lines. In particular, cells from different organisms can create extremely diverse glycan species as those synthesized by mammalian cells (including different linkages, glycan monomer compositions and structures). Thus, the complex PTM process, as glycosylation, has to be considered starting at the early stage of biopharmaceutical development, till the

administration to patients. The efficacy and safety for the patients has to be ensured, to avoid reduced efficacy or even undesirable immunogenicity [11, 12].

Glycosylation is highly complex. The amino acid sequence of glycoproteins exhibits one or more possible glycosylation sites with at least one covalently attached monosaccharide or oligosaccharide. However, not every potential glycosylation site is strictly occupied. In human proteins the most abundant sites are at asparagines (N-linked glycosylation in a motif of Asparagine-X-serine/threonine) and certain serine or threonine residues (O-linked glycosylation). The focus of glycosylation analysis in the presented work is set on N-glycosylation. N-glycosylation shows a high degree of microheterogeneity due to the complex pool of monosaccharides, differences in branching and antennae length. The different monosaccharides are very similar in structure, composition and molecular weight. The basis of all N-glycans is a core structure of two N-acetylglycosamines linked to three mannose residues. This trimannosyl core can be linked to further glycan monomers at the individual mannose residues, hereby 2-5 antennae can be formed [13]. According to their structure (monomer type, order and linkages), they are classified into three main groups. The most abundant are high-mannose and complex types. The high mannose type contains between five and nine mannose residues which are linked to the core structure. The complex type is often branched containing different glycan monomers in addition to mannose. Thereof, five typical complex-type N-glycans in monoclonal antibodies (G0F, G1F, G2F, A1F and A2F) [14, 15] were applied in this work. The structures are depicted in CFG (Consortium for Functional Glycomics) nomenclature in Fig. 1. The last one, not as common as the former types, is defined as hybrid type and combines the high mannose and the complex structure.

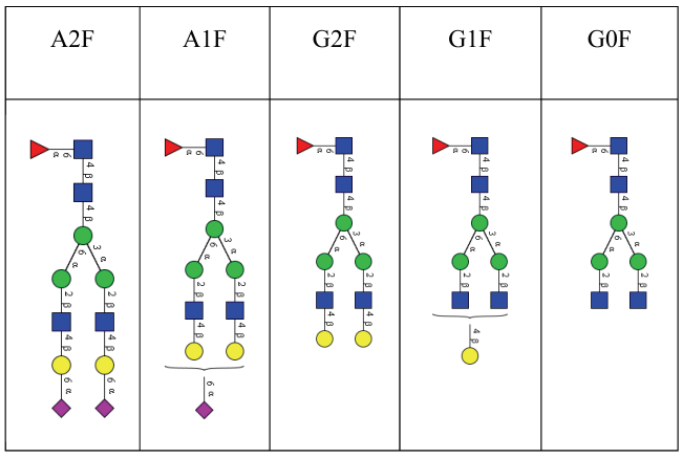

Fig. 1: Five complex type N-glycans abundant in many therapeutic antibodies. The glycan structures are displayed according to the CFG nomenclature: ■: N-Acetylgalactosamine, ● : Mannose, ●: Galactose, ▲: Fucose, ♦: N-Acetylneuraminic Acid

The analysis of glycosylation is challenging due to the described high macro- and microheterogeneity of N-linked glycans. Thus, the development of methods for the analysis of closely related glycoforms (i.e. including structural glycan isomers) is of high interest in the pharmaceutical industry as well as in the academic area. There are several techniques to encode this complex process. The most common techniques are described in several standard bioanalytics and biochemistry books as well as in a rapidly increasing number of review articles about glycoprotein analysis [16-20].

Glycosylation analysis can be performed on glycoproteins, glycopeptides and released glycans. The separation of glycoproteins is frequently performed with sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE), often in combination with isoelectric focusing in 2-dimensional gel electrophoresis. Carbohydrate-specific lectins can be used to sensitively detect and characterize the glycoforms of blotted proteins after gel electrophoresis. Alternatively, the selectivity of lectins is employed in lectin affinity chromatography [21, 22]. Almost complete or at least partial resolution of glycoforms can be obtained via capillary zone electrophoresis (CZE). However, the characterization of glycoforms is hardly possible. Thus, mass spectrometric characterization of glycoproteins can be performed additionally applying a matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) instrument.

Glycopeptide analysis enables the identification of site-specific glycosylation. For this purpose the glycoprotein is digested with specific (e.g. trypsin, Asp-N, Lys-C, or Glu-C) or nonspecific endoproteases (e.g. pronase) to obtain glycopeptides carrying individual glycosylation sites. Afterwards the peptides are separated, commonly by high performance liquid chromatography (HPLC) on reversed or normal phase. The glycan structures can be characterized by fragmentation experiments by electrospray ionization/mass spectrometric detection (ESI-MS/MS) using frequently collision-induced dissociation (CID) and/or electron transfer dissociation (ETD), via the exact mass difference of the glycan-released peptide to the glycopeptide.

However, both glycoprotein and glycopeptide analysis are hardly capable to analyze structural glycan modifications, minor changes of monomer composition or structural isomers. Furthermore, glycan analysis is universal for most glycoproteins, whereas methods for glycoprotein and glycopeptides might need modification (i.e. sample pre-treatment, suitable enzymes, separation methods etc.) depending on the individual glycoprotein. Therefore, glycan characterization based on glycoprotein-glycans is of high importance in biopharmaceutical and biological research [23]. The analytical strategies for glycoprotein-glycans range from different types of capillary electrophoresis (CE) and several types of liquid chromatography (LC) on the one hand to different optical and mass

spectrometric detection methods on the other hand [17, 24, 25]. The glycans are either enzymatically or chemically released from the protein or peptide moiety [26]. N-glycans are effectively and most commonly released by glycopeptide N-glycosidase (PNGase F). To enhance the efficiency of glycan cleavage the glycoproteins are often denatured by reduction with mercaptoethanol/SDS or dithiothreitol and then alkylated for example with iodoacetamide. Afterwards, the glycans can be separated by chromatographic or electromigrative techniques. Common chromatographic methods are hydrophilic interaction chromatography (HILIC) [27, 28], high-pH anion exchange chromatography (HPAEC) [29-32], reversed phase (RP)-HPLC and porous graphitic carbon (PGC)-LC [33, 34]. Electromigrative separation is mainly performed as CE next to microchip systems [35-39].

The CE technique is widespread in the biopharmaceutical field [17, 40-44]. Major strategies are CZE and capillary sieving electrophoresis (CSE, term is used according to IUPAC nomenclature [45]). LC and CE techniques often include the labeling of the reducing end of released glycans to enable separation or/and the following optical (fluorescence or UV) or MS [19, 46] detection. MS detection can be performed using ESI or MALDI. MALDI-TOF is suitable for neutral as well as charged glycans; however, fragmentation of fragile glycan compounds is likely [47]. ESI-MS is the method of choice in combination with most glycan separation techniques. The detection of charged, often labeled glycans in ESI-MS is more sensitive than MALDI and the extent of acidic glycan fragmentation is much lower. Both MALDI-TOF and ESI-MS provide information on glycan composition by accurate mass. More detailed information about glycan monomer linkages and branching can be obtained for native glycans using MS/MS fragmentation or repeated analysis after application of selective exoglycosidases.

Capillary electrophoresis is widespread in the pharmaceutical industry especially for routine analysis of glycosylation using CSE with laser induced fluorescence (LIF) detection. These systems provide fast analysis combined with high resolving power. Especially for a fast examination of glycosylation modifications these systems are well suited.

Glycans need derivatization prior to CE-LIF analysis to enable fluorescence detection and to enhance glycan separation [46]. Well studied ionic labeling reagents of common CSE-LIF methods are the negatively charged 8-aminopyrene-1,3,6-trisulfonic acid (APTS) [48-51], the 8-aminonaphthalene-1,3,6-trisulfonic acid [52-54] or 2-aminobenzoic acid [55, 56]. Cationic labeling reagents like the in HPLC commonly used 2-aminobenzamid can also be applied. However, the negative charge of attached sialic acids can form neutral or negatively charged glycans, which should be taken into account

during method development. Thus, negatively charged labels are generally preferred in CE for glycan analysis (enabling the analysis of uncharged and negatively charged glycans). Thereof, the trisulfonated APTS-label is one of the most abundant labels in routine CSE-LIF glycan analysis and was applied in this work. It provides fast analyte migration and high separation efficiency based on the analyte's size-to-charge ratio including the separation of structural isomers. Furthermore, commercially available analysis kits promote the widespread application. A first-hand identification or characterization of unknown glycan species by optical detection is not possible. Since standards are often not available or very expensive, alternative strategies are required. For this purpose CZE-MS is well suited because it combines the high separation efficiency of CE with accurate mass detection to identify glycan species. In this work APTS-labeled glycans as for CSE-LIF was used to enable the identification of LIF signals by CE-MS.

The following two paragraphs describe the theoretical and practical aspects of these two techniques.

1.1.1 Capillary Electrophoresis

CE is one of the most efficient techniques to separate ionic compounds. The principle of CE is based on the migration of ionic compounds in an electric field, whereby the analytes are separated with respect to their size-to-charge ratio. The term "Capillary Electrophoresis" involves different separation techniques such as CZE or CSE. CZE is the most common technique, often simply termed CE. Based on mobility differences the analyte ions are separated in capillaries filled with a homogeneous electrolyte. In CSE the capillaries are filled with high viscosity or polymerized electrolytes, frequently termed "capillary gel electrophoresis" (CGE). Here, the molecule size and structure have more influence on separation due to the sieving effect of the (partly) polymerized electrolyte.

The separation technique of choice for CE-MS is CZE [19, 57-61] because CZE enables superior separation efficiency based on the analyte's size-to-charge-ratio and is performed in MS-compatible background electrolyte (BGE) systems. Separation is mainly influenced by the electrophoretic mobility of the analytes in combination with the electroosmotic flow (EOF). The electrophoretic mobility is dependent on the charge of the analyte (influenced by the pH of the electrolyte), the analyte's size and the electrolyte's viscosity (temperature dependent).

The EOF is the motion of liquid created by the migration of net charge in the electrical double layer between capillary wall and electrolyte solution induced by an applied voltage at the capillary ends. The EOF velocity (v_{EOF}) is influenced by the applied field

strength E, the Zeta potential ζ (inversely proportional to the ionic strength of the BGE), the dielectric constant ε and the viscosity η of the electrolyte solution (equation 1).

(1) $$v_{EOF} = \frac{\varepsilon \times E \times \zeta}{4 \times \pi \times \eta}$$

The electroosmotic mobility (μ_{EOF}) is described by equation 2:

(2) $$\mu_{EOF} = \frac{\varepsilon \times \zeta}{4 \times \pi \times \eta} = \frac{v_{EOF}}{E}$$

Furthermore, the velocity of the EOF is a function of the temperature and of added organic solvents such as methanol. Both parameters affect the viscosity of the electrolyte and thereby the mobility of the EOF and the analytes. Alternatively, the EOF can be modified by capillary coatings as described in the next paragraph.

Capillary Coatings

The EOF velocity and direction can be modified by several static or dynamic capillary coatings, independent of the BGE's pH [62-64]. Hereby the EOF is enhanced, reduced, or even reversed, dependent on the coating's surface. In this work neutral coatings are applied to avoid creating an EOF at pH 4.0. The method is employed with reversed polarity (anodic detection), thus, the EOF runs toward the capillary inlet. In this way the method would be incompatible with MS-coupling because a suction into the capillary is created which affects the electrospray signal and the CE current. Consequently, hydrophilic nonionic polymers can be adsorbed, cross-linked or covalently bonded to the fused silica surface to significantly reduce or fully suppress the EOF.

1.1.2 Analysis of Negatively Charged Glycans

The CE-MS methods presented here are applied to negatively charged glycans (naturally by the presence of sialic acids or induced by a negatively charged label). There are two different principles of CE to selectively separate negatively charged analytes like APTS-labeled glycans.

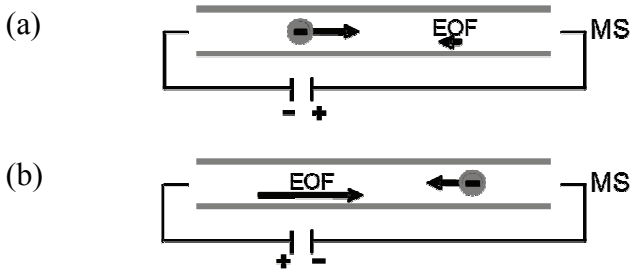

Fig. 2: CE separation techniques to separate negatively charged glycans by capillary electrophoresis/mass spectrometry. More details are described in the text.

The techniques are schematically displayed in Fig. 2, where:

(a) A negative potential is applied to the capillary inlet (reverse polarity) whereby the negatively charged glycans migrate to the anodic outlet. This technique requires an EOF close to zero. In combination with mass spectrometers a complete suppression of the EOF is required. The EOF can be suppressed using a BGE < pH 2, so almost all silanol residues are protonated. Alternatively, a neutral coating of the capillary inner wall avoids the generation of an EOF, as mentioned before.

(b) A positive potential is applied to the capillary inlet (normal polarity). A strong EOF (BGE at alkaline pH) transports the negatively charged glycans contrary to their inherent mobility to the cathodic outlet.

1.1.3 Mass Spectrometry

Common mass spectrometry methods applied in glycan analysis are based on ESI and MALDI techniques [65-67]. In this work capillary electrophoresis is used with online ESI-TOF-MS. Beside TOF instruments, other mass analyzers with medium or high resolution ($R \geq 5.000$) and fast scan rates are applicable.

Regarding the coupling with CE instruments, MS detectors with a grounded source and the electrospray potential applied to the entrance of the MS are clearly advantageous. These preconditions lead to a simpler method development, since the separation voltage can be applied independently to the electrospray process.

The next paragraph describes the principles of electrospray ionization and TOF detection techniques.

Electrospray Ionization

Electrospray ionization is a very efficient and soft ionization technique [68-71] which has made it very popular in analysis of bio-molecules. Solvated ions are transferred from liquid to gas phase in an electric field by desolvation with little energy transfer to the molecule. Between the MS entrance and the sprayer tip an electrospray potential is applied and by varying the polarity either positive or negative charges are generated. The solvated analytes are electrosprayed at the tip of a conductive sprayer needle as a fine aerosol, assisted by nitrogen flow. Contrary to MALDI, the formation of multiply charged ions is possible and enables the detection of small as well as large molecules like proteins. The amount of charges depends on the number of possible charge carriers and their positions in the molecule structure, e.g. APTS-labeled N-glycans create mostly doubly and triply charged ions. For CE-MS coupling a coaxial sheath-liquid (SL) interface is frequently used [72]. The SL closes the electric circuit for the inner capillary electrophoretic separation. Additionally, the SL, containing a mixture of organic solvents (e.g. 2-propanol or methanol) and water, supports the low flow in CE (nL/min range) to enable a stable spray and a good desorption of the formed droplets. Additives in the SL can support the ionization process, e.g. volatile acidic components like formic or acetic acid. During the electrospray process the charge density of the formed droplets increases while the solvents evaporate. The formation of free analyte ions in the gas phase is explained by two basic models [73]: the charged residue model of Dole or Röllgen's "single ion in droplet theory" and the ion evaporation model of Iribane and Thomson.

1.1.4 Time-of-flight mass spectrometry

The principle of MS is the separation of gas phase ions according to their mass-to-charge (m/z) ratios in an electric field. One efficient mass spectrometer is the TOF mass spectrometer. The basic principle of mass determination is based on very precise time-of-flight measurements of ions accelerated in a high vacuum. After the generation of gas phase ions in the ion source as described in the latter paragraph, the mass analyzer separates the generated ions according to their m/z ratios. The detector records at all times the intensity of the ions as a function of their m/z ratio, based on this the evaluation unit creates a mass spectrum. There are various techniques of ion generation and mass separation [74].

The generated ions are focused and transferred to the flight tube by several lense arrangements. Hybrid QTOF mass spectrometers include an analytical quadrupole and a gas filled quadrupole acting as a collision cell in front of the time-of-flight tube. Hence, selective ion fragmentation can be carried out for the identification of unknown

substances or the verification of putative sum formulas. Finally the ions are pulsed orthogonally accelerated into the field-free tube with a single acceleration voltage of several kV. Depending on their m/z ratio the different ion velocities arise. During the flight in the field-free space the ions are separated. At the upper end of the tube a reflector directs the ions back to the detector. The accurate m/z ratio is precisely determined out of the fixed flight path s (1-4 m), the flight time t (from a few to 100 µs), the elementary charge e and the acceleration voltage U (equation 3).

(3) $$\frac{m}{z} = \frac{2 \cdot e \cdot U \cdot t^2}{s^2}$$

The main advantages of TOF-MS are the high resolution (up to 10,000) and the high accuracy of mass determination (up to 1 ppm) in addition to a large dynamic range and high speed detection. The instrument used in this work is equipped with an analogue to digital converter (ADC) enabling a discrimination free measurement of the isotopic pattern.

The coupling of CE with an ESI-TOF mass analyzer is an ideal combination for the analysis of anions in complex matrices. The fast, high resolving detector is able to separately detect substances with very similar migration times and can identify them by accurate mass.

1.2 Aims and Scope

The aim of this work is to establish CE-MS methods for glycan identification and characterization in the biopharmaceutical field. State of the art for routine as well as high-throughput glycan analysis is CSE-LIF using 8-aminopyrene-1,3,6-trisulfonic acid (APTS) labeled glycans. Since first-hand identification of unknown glycans is not possible by optical detection an alternative method is required. CE-TOF-MS presents a powerful and selective orthogonal technique to ease the identification. Glycan analysis by CSE-LIF provides high separation efficiency (i.e. separation of structural isomers) and excellent detection sensitivity for APTS-labeled glycans. Thus, the request for CE-TOF-MS is set on high level. CE-MS methods with high separation efficiencies similar to CSE are rare in this field [19, 59, 75-77] (manuscript 1).

CE-MS method development was primarily based on the objective to separate structural glycan isomers. Therefore, the two structural isoforms of G1F were chosen as a reference. Furthermore, the methods were supposed to be as comparable as possible to

common CSE-LIF methods. Thus, this work was focused on APTS-labeled glycans, due to their direct compatibility to common CSE-LIF systems. The applied separation voltage is favored to be in reverse mode with anodic detection, to obtain the same migration direction as CSE and enables a direct comparability of glycan patterns obtained by CSE-LIF and CE-MS (manuscript 3).

In this work two different CE-MS methods for the identification of glycans are presented. Besides an acidic BGE system, with the favored anodic detection, an alternative alkaline BGE system with cathodic detection and hence reverse migration order is proposed. Both methods have been optimized and validated for the application to biopharmaceutical samples. The methods' comparability with standard CSE-LIF systems has been evaluated (manuscript 2 and 3).

Finally the reduced detection sensitivity of TOF-MS compared to highly sensitive fluorescence detection was to be solved, since the determination of minor glycan species found by LIF detection is of importance. An online CE-LIF-MS setup has been implemented. Additionally, an in-line solid phase extraction (SPE)-CE-MS setup has been developed to concentrate the analytes after injection, prior to separation (manuscript 4).

Chapter 2

Manuscripts

Manuscript 1:

M. Pioch, S.-C. Bunz, C. Neusüß:

Capillary electrophoresis/mass spectrometry relevant to pharmaceutical and biotechnological applications.

Electrophoresis 33(11), 1517-30 (2012)

Manuscript 2:

S.-C. Bunz, F. Cutillo, C. Neusüß

Analysis of native and APTS-labeled N-glycans by capillary electrophoresis/time-of-flight-mass spectrometry

Analytical and Bioanalytical Chemistry, 405 (25): 8277-8284 (2013)

Manuscript 3:

S.-C. Bunz, E. Rapp, C. Neusüß

Capillary electrophoresis/mass spectrometry of APTS-labeled glycans for the identification of unknown glycan species in capillary electrophoresis/laser induced fluorescence systems

Analytical Chemistry, 2013, DOI: 10.1021/ac401930j

Manuscript 4:

K. Joos, J. Sommer, S.-C. Bunz, G. K. E. Scriba, C. Neusüß

In-line SPE-CE-MS using a fritless bead string design – Application for the analysis of organic sulfonates including APTS-labeled glycans

Electrophoresis, 2013, DOI: 10.1002/elps.201300388

Manuscript 1

Capillary electrophoresis/mass spectrometry relevant to pharmaceutical and biotechnological applications

M. Pioch, S.-C. Bunz, C. Neusüß
Electrophoresis 33(11), 1517-30 (2012)

The manuscript reviews the actual developments of CE/MS in the biopharmaceutical field. The glycosylation analysis is presented based on glycoproteins, -peptides as well as released glycans. Recent approaches and applications for carbohydrate analysis are presented starting from 2006.

Candidate's work:	Literature research and review preparation of introduction parts and preparation of the carbohydrate section.

Electrophoresis 2012, *33*, 1517–1530

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Review

Capillary electrophoresis/mass spectrometry relevant to pharmaceutical and biotechnological applications

Advanced analytical techniques play a crucial role in the pharmaceutical and biotechnological field. In this context, capillary electrophoresis/mass spectrometry (CE/MS) has attracted attention due to efficient and selective separation in combination with powerful detection allowing identification and detailed characterization. Method developments and applications of CE/MS have been focused on questions not easily accessible by liquid chromatography/mass spectrometry (LC/MS) as the analysis of intact proteins, carbohydrates, and various small molecules, including peptides. Here, recent approaches and applications of CE/MS relevant to (bio)pharmaceuticals are reviewed and discussed to show actual developments and future prospects. Based on other reviews on related subjects covering large parts of previous works, the paper is focused on general ideas and contributions of the last 2 years; for the analysis of glycans, the period is extended back to 2006.

Keywords:
Capillary electrophoresis/mass spectrometry / Glycosylation / Intact protein analysis / Pharmaceutical applications
DOI 10.1002/elps.201200030

1 Introduction

Pharmaceutical development and production require adequate analytical techniques. Especially in research and development as well as in all aspects of the strongly growing biopharmaceutical sector, advanced characterization tools are required. Mass spectrometry plays a crucial role as it allows the rapid and sensitive characterization based on (accurate) mass measurement or tandem mass spectrometry [1–6]. However, separation is generally required in order to gain analyte capacity, matrix removal, and/or to avoid ionization suppression. Liquid chromatography primarily in the reversed-phase mode is mainly used as a separation technique due to its robust and reproducible set-up, its wide applicability based on hydrophobic interaction, and its straightforward combination with electrospray ionization mass spectrometry [7–9]. However, electrophoretic separation is widespread for the analysis of biopolymers, for example, for DNA sequencing or protein analysis by SDS-PAGE [10, 11]. The capillary dimension allows zone electrophoretic separation and, thus, applications like the separation of stereoisomers where CE is mostly applied in the pharmaceutical industry [12]. More important, direct coupling to mass spectrometry is feasible in this way. CE/MS has developed in recent years toward a routinely applicable technique [13–17]. Many pharmaceutical companies have invested in this technique in the last years.

Generally CE is beneficial with respect to separation efficiency and short analysis time [18, 19]. The injection of small sample amount in CE (compared to LC) is an advantage in certain cases, whereas, it also leads to limited concentration sensitivity which is a general drawback of CE. However, in the pharmaceutical context, sufficient and high concentrated samples are often available. The main reason applying CE and CE/MS is its selectivity even if additives like sieving matrices ("gels") or nonvolatile BGE additives like cyclodextrins (separation of enantiomers) interfere with the analytes in the ESI process (and lead to contamination of the MS). Thus, almost all applications of CE/MS in the pharmaceutical area are based on CZE separation. CZE/MS is suitable to analyze all kind of ionic molecules. Both acidic and basic analytes are accessible, separating and detecting the analytes as cations or anions. Small molecules as well as amino acids or peptides

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Abbreviations: **2-AA**, 2-aminobenzoic acid; **2-AB**, 2-aminobenzamid; **AGP**, alpha-1-acid glycoprotein; **AHL**, N-acyl-L-homoserine-lactonase; **APTS**, 9-aminopyrene-1,3,6-trisulfonic acid; **CSE**, capillary sieving electrophoresis; **DMA-EpyM**, acrylamide-pyrrolidine methacrylate; **DS**, dextran sulfate; **EPO**, erythropoietin; **EtOH**, ethanol; **FA**, formic acid; **GH**, growth hormone; **Hac**, acetic acid; **hGH**, human growth hormone; **IPA**, isopropyl alcohol; **MeOH**, methanol; **NH_4Ac**, ammonium acetate; **PVS**, poly(vinyl sulfonic acid); **rhGH**, recombinant human growth hormone; **rhIFN-β**, interferon-β-1a; **rMAb**, recombinant monoclonal antibody; **SOD**, superoxide dismutase

Colour Online: See the article online to view Fig. 1 in colour.

have been analyzed in this way [17, 20, 21]. Intact protein analysis has developed to a major application of CE/MS in the last years [22, 23]. Appropriate coating of the capillary wall facilitates efficient separation among proteins, protein isoforms, or matrix (e.g. salts) [24–32].

Glycosylation studies are of great interest in the pharmaceutical area [33, 34]. Many biopharmaceuticals are glycoproteins, for example, antibodies are generally glycosylated. As glycosylation influences the protein folding, protein stability, and the biological activity, protein glycosylation profiling is important in the field of product development and quality control [35]. Glycosylation analysis can be performed on intact protein level, on the peptide level, i.e. after proteolytic digestion of the protein, or on the level of the glycans. All three approaches can be performed using CE/MS. However, CE/MS is especially suitable for the analysis of intact proteins [22] and the analysis of glycans [36] due to the separation selectivity of CE based on charge/size ratio of the analytes. Glycopeptides are primarily analyzed by LC/MS, however, the separation of glycopeptides from nonglycosylated peptides by CE/MS can be an advantage [37].

Here we summarize recent publications in the method development and the application of CE/MS relevant in the pharmaceutical context. Several application fields of CE/MS in the pharmaceutical area are included; however, we focus on the analysis of intact proteins and the analysis of glycosylation. As several reviews cover parts of this subject mainly studies published in the last 2 years are summarized here, for the analysis of glycans the period is extended back to 2006.

2 Technical aspects

2.1 Capillary electrophoresis

CZE is the key mode of capillary-based electromigration techniques when coupled to mass spectrometry. However, MEKC [38, 39], CGE or CSE [40, 41], CEC [42–44] have been coupled to MS in the last years. Nevertheless, if any electromigration technique is expected to be routinely applied to pharmaceutical applications, CZE/MS is the method of choice. This is obvious from the summary of the recent papers as presented in the following parts of this review article. Nonaqueous CE/MS (NACE/MS) is an interesting alternative to achieve a certain selectivity or to enable the analysis of hardly water-soluble analytes [45]. A further increase in applications of NACE/MS also in the pharmaceutical context is expected. Any CE instrument can be used for CE/MS as long as the capillary can be led out of the instrument and the software accepts external grounding of the capillary outlet.

2.2 Interfaces

Electrospray ionization is the technique of choice for the transfer of the ions from the end of the separation capillary into the mass spectrometer. The most robust and most widely used approach is the use of a coaxial sheath-liquid interface[46]. CE/MS method development is straightforward if the interface is on ground and the inlet of the MS is put onto high voltage for ESI. By means of the used sheath liquid additional acidity (especially for intact protein application) or basicity can be introduced to improve the ionization efficiency. The relevant interface parameters are listed in the Tables 1–3 of this review.

Nanoelectrospray is of interest due to increased sensitivity, however, mostly lacks robustness or is useful for certain applications only. An interesting approach is based on the development of a porous sprayer [47]. A recently developed prototype has been applied for peptide analysis [48, 49] and intact protein analysis [50]. S/N ratio has been reported to be increased by more than one order of magnitude for small model proteins. The versatility and ability for routine and robust application, however, still needs to be shown. A miniaturization of the sheath-liquid interface is an interesting alternative [51, 52]. Maxwell et al. applied such an interface to the analysis of glycans [53]. However, a thorough comparison to alternative other interfaces remains to be performed.

APCI or APPI might in certain cases be an option, if the BGE contains nonvolatile constituents [54, 55] or additional selectivity (nonionic analytes) is required [56]; however, ESI is usually by far more efficient for ionic analytes as usually analyzed by CE.

2.3 Mass spectrometry

The mass spectrometer provides the required selectivity and mass accuracy for each application. All kinds of mass analyzers can be used for small molecule analysis by CE/MS. Tandem mass spectrometry can be beneficial for peptide sequencing, structure analysis, or the gain of selectivity, while high resolution provides also enhanced selectivity and is the basis for mass accuracy, potentially enabling the determination of the elemental composition. The concepts, instrumental considerations, and applications of CE/TOFMS have been recently reviewed [57].

Ion traps are often used for the analysis of peptides and small pharmaceutical substances [9, 12, 15], while TOFMS was used due to its higher mass accuracy [8]. Small pharmaceutical substances in blood serum were analyzed by TOFMS to provide enhanced selectivity [11, 14]. Common mass analyzers in the field of CE/MS for glycan analysis are TOF, QTOF, and ion trap instruments.

In case of the analysis of intact proteins mostly TOFMS was employed, providing the necessary mass range for the macromolecules and the short spectrum acquisition time for the peak plotting. One exception is a study where an ion trap was sufficient to distinguish between the monomeric and dimeric form of a metal-protein complex [58]. Modern benchtop TOF-MS instruments enable the resolution of proteins up to about 10–20 kDa, whereas high-resolution TOF-MS instruments and orbitrap instruments can resolve proteins up to about 30 kDa [59, 60].

Table 1. Examples of CE/MS methods and applications for the analysis of small pharmaceutical substances and peptides in the years 2010 and 2011

Analyte	Coating	BGE	SL	MS	Application	Citation
Imatinib (cancer drug)	M7C4I	NH_4Ac 30 mM	ACN/H_2O (50/50 v/v)+10 mM HAc	TOF	Quantitative analysis in human serum	[83]
Tricyclic antidepressants	M7C4I	30 mM NH_4Ac at pH 3	ACN/H_2O (50/50 v/v)+10 mM HAc	TOF	Drug quantification in human plasma, pharmaceutical formulations	[82]
Bioactive peptides	-	1 M HAc	CE-IT-MS: IPA/H_2O (60/40 v/v) + 0.05% v/v FA CE-TOF-MS: same with diluted reference substances	Ion trap, TOF	Peptide identification in enzymatic bovine milk digest	[77]
Substance P, its aminoacids	-	25 mM HAc+25 mM FA pH 2.5–5.0	50% MeOH + 0.1% FA	Ion trap	Peptide hydrolyzation characterization in cell culture	[65]
Rosiglitazone metformin	-	50 mM FA pH 2.8	MeOH/H_2O (50/50 v/v) + 0.5% v/v FA	QTOF	Detection in human serum	[81]
Tetracosactide	-	0.5 M FA	H_2O/IPA (50/50 v/v)	QTOF	Impurity detection in drug	[79]
D-Carnitine	-	0.2% Succ-γ-CD in 0.5 NH_4FA	IPA/H_2O (50/50 v/v) + 0.1% v/v FA	Ion trap	Chiral analysis of pharmaceutical formulation	[129]

3 Amino acids, peptides, and small molecules

In the analytical field of small pharmaceutical molecules, the most often employed technique is HPLC, due to its robustness, reproducibility, and wide experience of analysts with this technique. However in the recent years, CE applications were implemented as a complementary or orthogonal technique due to the different separation principles and the possibility to solve complex analytical problems, which are for example reviewed by Suntornsuk in 2010 [61]. As can be seen in the review, most CE methods employ optical detectors like UV and DAD; however, the detection by ESI-MS is slowly rising. Examples for this development are shown in Table 1 for 2010 and 2011.

A large part of pharmaceutical substances contain nitrogen in form of an amine group (e.g. amino acids, peptides, alkaloids, etc.). These analytes are mostly separated as cations in an acidic BGE based on formic or acetic acid, possibly buffered with ammonium as counter ion. An extensive summary of BGEs for CE/MS is presented by Pantuckova et al. 2009 [62].

Amino acids can be directly analyzed by CE/MS [63, 64] using acidic BGEs, mainly based on formic acid. The majority of the published applications deal with metabolism studies or clinical applications [65–69] or the analysis of amino acids in food [70].

CE/MS is often applied to the analysis of peptides [71], however, most applications can be found in the context of biological, metabolism, or clinical issues. As this application area is rather large and so far only of minor interest in the pharmaceutical context, we refer to existing reviews [72–76]. Several recent studies deal with the identification of peptides in complex biological samples, as bovine milk protein hydrolysate [77] and enzymatically digested human cell cultures [65]. In both cases, bare fused-silica capillaries with an acidic BGE were hyphenated via ESI-interface to an Ion trap. Stanova et al. [78] analyzed the therapeutic peptides anserine, carnosine, and buserelin in human urine. Preparative ITP was used as sample preparation method increasing the CE/MS sensitivity (high ppb range) by factors higher than 25.

CE/MS is well suited for impurity profiling of drugs, as recently demonstrated by our group for the peptidic drug tetracosactide [79]. Figure 1 shows the comparison of CE/MS and LC/MS for the characterization of impurities of this 24 amino acids containing peptide. CE/MS turned out to be better suited than LC/MS for the characterization of peptide fragments (incomplete synthesis). However, LC/MS showed a better separation of peptides containing protecting groups, as these modifications increase substantially the hydrophobicity of the peptides. This example demonstrates the often observed complementary characteristic of CE/MS and LC/MS for the analysis of complex peptide mixtures [48, 80].

CE/MS is useful for the characterization of pharmaceutically relevant small molecules. Bare fused-silica capillaries and a BGE of formic acid at pH 2.8 were used for the analysis of the diabetes drugs rosiglitazone and metformin in human blood serum [81]. The resulting method reached an LOD for rosiglitazone of 4.4 ng/mL and 2.1 ng/mL for metformin, sufficient for monitoring therapeutically relevant serum concentrations.

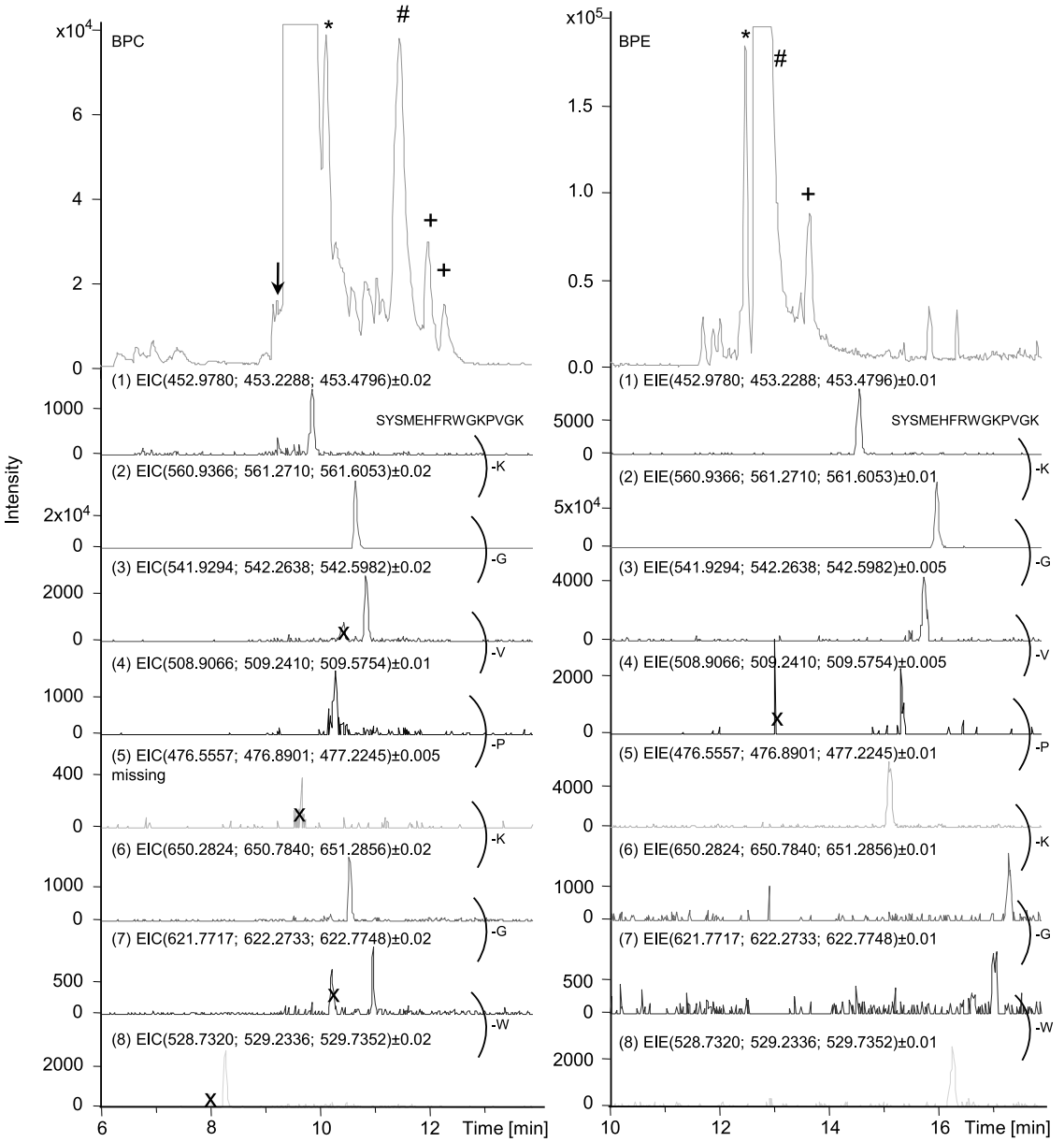

Figure 1. TCS sample analysis by RP-HPLC-MS (left) and CE-MS (right): BPC, BPE and ion traces of selected peptides smaller than TCS (fragments 1–15 TCS, 1–14 TCS, 1–13 TCS, 1–12 TCS, 1–11 TCS, 1–10 TCS, 1–9 TCS, and 1–8 TCS). EICs and EIEs were created by extraction of the masses of the three main isotopes of the most abundant charge state of the respective peptide (listed in the figure). The extraction width was adapted in order to exclude contaminants or isotopes of other ions showing the same nominal mass. The letters indicate the missing amino acid from one peptide to the following starting with the peptide SYSMEHFRWGKPVGK. The TCS itself is the peak being out of range, the asterisk indicates the 16D-Lys-TCS diastereomer, the arrow indicates TCS sulfoxide, the number sign indicates TCS+tBu (not separated from TCS by CE/MS), the plus indicates TCS+tBoc. X denotes peaks arising from background substances or isotopes of comigrating peptides lying within the specified extraction width. Modified from Taichrib et al. 2011 [79]

In some cases, the application of a capillary coating is also an advantage for the analysis of small pharmaceutical substances, in order to decrease peak tailing and peak asymmetries. Elhamili et al. [82, 83] investigated the use of ω-iodo-alkyl ammonium salt (M7C4I) coating for the analytic of tricyclic antidepressants and the anticancer drug imatinib in human plasma. In both applications, a BGE containing ammonium acetate and a sheath liquid of 50% ACN with

10 mM HAc was used, leading to high separation efficiencies and migration time repeatabilities. A sample preparation step using a strong cationic SPE (SCX-SPE) for the extraction of small basic analytes with restricted access for proteins, enables the analysis, quantification, and clinical monitoring of tricyclic antidepressants and imatinib within blood serum or pharmaceutical formulations.

The separation of enantiomeric analytes is a major application of CE in the pharmaceutical area [12, 84], especially for the determination of enantiomeric purity. However, CE/MS is not often applied in this field as (i) the applied enantioselectors (cyclodextrins) yield ionization suppression and (ii) the analytes are usually well known. We refer for this subject to a recent review about CE/MS for the analysis of chiral analytes [85]. By the way, charged cyclodextrins can be characterized by CE/MS [86].

4 Intact proteins

4.1 Methods for intact protein analysis

The methods developed for intact protein analysis were further refined in the last few years and several applications which are of potential interest for the analysis of (bio)pharamaceuticals have been developed and investigated. Based on previous summaries of intact protein analysis by CE/MS [22, 23, 87], a short overview of analytes, BGE, sheath liquid, and application with respect to intact protein analysis by CE/MS is given in Table 2. Important aspects are discussed in the frame of previous publications in the following sections.

4.1.1 BGE

The same BGEs used in CE/MS for peptides and small drugs can in principle also be used for the analysis of intact proteins. Although ammonium formate is used in several studies, the ammonium ion might result in an additional heterogeneity of the charge distribution in the ESI, which might diminish the overall signal intensity of the protein. Thus, acetic or formic acid are mainly used as BGE for intact protein analysis by CE/MS. Acetic acid seems to be advantageous when larger proteins or glycoproteins are analyzed. Formic acid provides a lower pH, which might be beneficial for acidic proteins and in order to obtain a charge distribution at higher charge states, i.e. at lower m/z values.

4.1.2 Prevention of protein–capillary interaction

A major problem of capillary electrophoresis for the analysis of proteins/peptides is the potential adhesion of these substances to the fused-silica capillary wall [28], since it influences the number of theoretical plates, peak width, and thus the resolution. It can even lead to complete loss of analyte signal at the detector. Additionally, protein adhesion from previous runs influences the repeatability of the EOF and the migration times. The process of adhesion from protein to the fused-silica wall is governed mainly by charged residues in the protein, which first contact to the opposite charged capillary wall. The adhesion processes are governed mainly by electrostatic or hydrophobic interactions. For an extensive discussion of the process of protein adhesion, see a review from Stutz [28].

The main strategies to prevent protein adhesion have primarily focused on the prevention of first contact between the protein and the fused-silica surface of the capillary, either by a strong acidic or basic BGE, coating of the capillary wall or by the use of BGE additives. The latter is generally not recommended when ESI-MS is used as detector for CE.

4.2 Bare fused-silica capillary

In some papers, bare fused-silica capillaries were used for the analysis of proteins. In order to minimize the protein adhesion, mostly strong acidic or basic BGEs are required. For example Staub et al. [88] used 75 mM ammonium formate at a pH of 9.5 for the separation of Oxyglobin® from hemoglobin, with a migration time RSD smaller than 2% ($N = 10$).

The use of strong acidic or basic BGEs, however, often results in protein denaturation and/or protein degradation. Borges-Alvarez [58] compared the usability of a 1 M HAc buffer at pH 2.3 with a NH_4Ac buffer at pH 7.3 for the analytics of bovine Cu, Zn-superoxide dismutase. In its native form, the dismutase occurs as a Cu_2Zn_2-dimer, which resulted in a single sharp peak when using 10 mM NH_4Ac (pH 7.3). The use of the acidic HAc, however, resulted in two distinct peaks with the main peak close to the mass of the protein monomer, showing that the native enzyme was denaturated by the acidic conditions.

4.3 Use of organic modifiers

Organic modifiers can be added to the BGE altering the hydrophobic properties of the protein for further decrease of protein adsorption. This principle was used in a study by Staub et al. [89] for the separation of human growth hormone (hGH) from the recombinant form (rhGH). The previously used basic BGEs, leading to protein degradation and some protein adsorption (and thus instable migration times), were replaced by an acidic BGE. In order to prevent adsorption, use of organic solvents (ACN, MeOH, and EtOH) in the range of 5–80% v/v were investigated via CE-UV. The best results regarding adsorption reduction and peak stability were achieved using a BGE consisting of 75 mM ammonium formate with 20% ACN. After optimization of the ESI source parameters via design of experiments, the developed method could be deployed in CE/TOFMS, using IPA/H_2O/FA (50:50:0.05 v/v/v) as sheath liquid. With the developed method, the distinction of hGH from rhGH was possible via electrophoretic mobility differences and differences in the isoform

Table 2. CE/MS methods and applications for the analysis of intact proteins in the years 2010 and 2011

Analyte	Coating	BGE	SL	MS	Application	Citation
α-Chymotrypsinogen A, ribonuclease A, lysozyme, cytochrome c	PB-DS-PB	50 mM HAc pH 3	IPA/H_2O (75/20 v/v) + 0.1% v/v HAc	TOF	Method development	[94]
RhGH	PB-PVS	75 mM NH_4FA pH 8.5	ACN/H_2O (75/25 v/v) + 5% v/v FA	TOF	Characterization of biopharmaceutical formulations	[93]
Oxytocin	PB-DS-PB	525 mM HAc pH 3	IPA/H_2O (75/25 v/v) + 0.1% v/v HAc			
rhIFN-β	PB-DS-PB	50 mM HAc pH 3	IPA/H_2O (75/25 v/v) + 0.5% v/v HAc			
Drug-lysozyme conjugates	PEI	100 mM HAc pH 3 + 5% v/v IPA	Sheathless porous sprayer	TOF	Characterization of drug conjugate	[96]
Insulin, ribonuclease A, carbonic anhydrase, lysozyme	PEI	100 mM HAC pH 3 + 5% v/v IPA	Sheathless porous sprayer	TOF	Method development	[50]
Intact glycosylated AGP	DMA-EPyM	1 M HAc	IPA/H_2O (50/50 v/v) + 1% v/v HAc	QTOF	Glycoprotein characterization in human serum, enrichment via antibody column	[97]
Deglycosylated protein	LN	1 M HAc	IPA/H_2O (50/50 v/v) + 1% v/v HAc			
rhGH	-	75 mM NH_4FA pH 2.5 + 20% ACN	IPA/H_2O (50/50 v/v) + 0.05% v/v FA	TOF	Identification of rhGH in lyophilized powder derived from Doping Analysis Lab	[89]
Hemoglobin-based oxygen carriers (HBCO)	-	75 mM NH_4FA	IPA/H_2O (50/50 v/v) + 3% v/v FA	TOF	Distinction of human Hb and oxyglobin (polymerized bovine Hb) in human plasma	[88]
Recombinant human insulin	-	75 mM NH_4FA pH 9.0 + 10% ACN	IPA/H_2O (50/50 v/v) + 1% v/v FA	TOF	Quantification, profiling in pharmaceutical formulation	[130]
Bovine CU, Zn superoxide bismutase	-	1 M HAc (10 mM NH_4Ac pH 7.3 also tested)	IPA/H_2O (60/40 v/v) + 0.5% v/v FA	Ion trap	Characterization of metal-protein complexes	[58]
AHL-lactonase	Dynamic polybrene	10 mM NH_4Ac + 0.1% polybrene pH 6.9	Sheathless porous sprayer	QTOF	Metal stoichiometry of protein-metal complexes	[109]
Growth hormone	-	75 mM NH_4FA pH 2.5 + 20% ACN	IPA/H_2O (50/50 v/v) + 0.05% FA	TOF	Method development	[90]
Hemoglobin		75 mM NH_4FA pH 9.0	IPA/H_2O (50/50 v/v) + 3% v/v FA			
Insulin		75 mM NH_4FA pH 9.0 + 10% ACN	IPA/H_2O (50/50 v/v) + 1% v/v FA			
rhGH	-	40 mM NH_4HCO_3 pH 8.5	IPA/H_2O (1:1 v/v) + 0.2% v/v FA	QTOF	Biopharmaceutical formulations	[59]
EPO	LN	1M HAc	IPA/H_2O (1:1 v/v) + 1% v/v HAc			

distribution: hGH contains a 20 kDa and a 22 kDa isoform, while rhGH only contains the 22 kDa isoform. In a subsequent study, the effects of reversible and irreversible adsorption were studied on the three model proteins growth hormone (GH), hemoglobin (Hb), and insulin (INS) [90]. As estimates for reversible adsorption, the variance of the migration times after successive analyte injections was used, while peak area recoveries and EOF conservation were used

to estimate the irreversible adsorption. The results showed that the addition of 10% to 20% ACN improved the peak area recoveries of insulin and growth hormone. However in case of hemoglobin, the peak area recovery decreased from 100% to 82%, while the variance of the migration time strongly increased from 1.7% to 19.7%, showing that both reversible and irreversible adsorption occurred by addition of ACN. It can thus be concluded that the addition of organic modifiers may be beneficial for the separation, but has in some cases deteriorating effects on the peak area recovery and migration time variance. For this reason, the addition is to be checked individually for each protein.

4.4 Coatings

An often used strategy to prevent protein wall adsorption is the use of capillary coatings. In the last several years, a wide variety of different coatings have been developed for the use in CE-UV [24, 29, 30, 32, 91, 92]. Charged coating can suppress electrostatic interaction by repulsion of the protein from the identically charged capillary wall. In case of neutral coatings, the access to the silanol surface is minimized. Using charged coatings, the direction and magnitude of the EOF can be chosen according to the analytical problem in order to increase the peak resolution, or to speed up the analysis if sufficient resolution is already achieved. Despite longitudinal diffusion, which is small in liquids, the resolution is improved if the analytes migrate as slow as possible, which can be accomplished by using an EOF working in the opposite direction than the migration direction of the analytes. The magnitude of the EOF has then been chosen (e.g. pH of the BGE) in such a way that the analytes still reach the detector. Another aspect of capillary coating is the deployment onto the capillary wall. In case of dynamic coatings, the coating agent is contained in the BGE that continuously regenerates the coating surface. Static coatings are applied via rinsing with coating agent prior to the analysis. Frequent recoating between runs is often required to guarantee reproducibility.

In CE/MS, only a small subset of the developed strategies for CE/UV can be used, since some coating agents are detrimental for the MS or the ionization process [25]. For example, dynamic coatings are seldom used in CE/MS since the coating agent in the BGE may suppress analyte signal. The choice of coating type is also limited, since at least a low BGE flow toward the ESI source is preferred to prevent sheath liquid from entering the capillary, otherwise current instabilities would impair the robustness of the method. The use and limitations of different coatings and CE modes for CE/MS has been recently discussed [25]. The following important contributions in this field from the last 2 years are presented and discussed.

Haselberg et al. investigated the use of a polybrene-based coating for the use in CE/MS [93, 94]. A bilayer coating consisting of polybrene and poly(vinyl sulfonic acid) (PVS) has already been used by Catai et al. in 2007 [95], which is only applicable for acidic proteins, since positively charge proteins bind to the negatively charged PVS. In order to circumvent this problem for basic proteins, a triple layer of polybrene–dextran sulfate (DS)–polybrene has been investigated on the proteins α-chymotrypsinogen A, ribonuclease A, lysozyme, and cytochrome c. The coating can simply be applied by subsequent rinsing of the capillary with PB solution/DS solution/PB solution with washing steps with deionized water in between. A BGE of 50 mM HAc at pH 3 and a sheath liquid consisting of IPA/H_2O/HAc (75/25/0.1 v/v/v) were applied. Measurements with BGE and a control experiment containing coating agents showed that the coating does not lead to any background signals in the mass spectra. Addition of polybrene to the BGE showed that low concentrations of polybrene results in no significant ion suppression behavior for the model proteins ribonuclease A and lysozyme.

Based on this work, the usefulness of polybrene multilayer coatings for the analysis of biopharmaceuticals was further investigated [93]. In order to test the capabilities of the bilayer PB-PVS coating for acidic proteins, heat-exposed rhGH was chosen as model protein. For the triple layer PB-DS-PB basic analytes, i.e. the nonapeptide oxytocin and its degradation products as well as the glycoprotein recombinant human interferon-β-1a (rhIFN-β) were chosen. For ideal separation and ionization of each model protein, different BGEs and sheath liquids were applied. The resulting methods allow the separation of analytes with modifications leading to charge differences from the original protein/peptide.

Another positively charged coating applied by Haselberg et al. [50, 96] was polyethylenimine (PEI). Since a sheathless interface (porous sprayer) was employed, an EOF from the inlet to the ion source stabilizes the ESI spray, which was achieved by the PEI coating in conjunction with a BGE consisting 100 mM HAc + 5% v/v IPA.

For the analysis of the intact human alpha-1-acid glycoprotein (AGP), Ongay and Neusüß [97] used the positively charged acrylamide-pyrrolidine methacrylate coating (DMA-EPyM), which was introduced by González et al. [98]. AGP presents a challenge due to its high heterogeneity based on five glycosylation sites and different amino acid sequences. The CE method used reversed polarity with a strong EOF toward the detector, which allowed the separation of the isoforms into an overlapping pattern of differently charged (different number of sialic acids) isoforms. In case of the deglycsosylated AGP, the DMA-EPyM coating could not be used, since the high-positive charge of deglycosylated AGP would cause migration toward the inlet vial overcoming the moderate anodal EOF. Instead of a cationic coating, a commercially available soluble polyacrylamide precoating was used. This neutral coating exhibits almost no EOF at low pH values. A good resolution was obtained through the resulting slow migration of AGP, leading to a separation of differences of the AGP backbone due to expression variants. The same soluble polyacrylamide coating was also used in the characterization of erythropoietin (EPO) by Taichrib et al. [59], where an excellent separation of EPO glycoforms is shown. Separation is not only obtained due to charge

differences (sialic acids) but also due to size of the glycans (antennarity).

4.5 Applications of intact protein analysis

A major application of intact protein analysis with CE/MS is the profiling and characterization of protein isoforms, which reveal information about charge differences, amino acid sequence differences, protein degradation, glycosylation heterogeneity, and other protein modifications. These differences are especially important in the biopharmaceutical field, since undetected protein forms or conformations are potentially harmful for patients and thus have to be monitored in quality control. The use for profiling has been demonstrated in various papers with the model protein rhGH. Staub et al. [89] showed the possibility to distinguish endogenous hGH from recombinant hGH. Catai et al. used a bilayer-coated capillary and ion trap MS detection to analyze rhGH [99]. They separated several forms of rhGH, postulating deamidation. Taichrib et al. were able to prove this hypothesis due to isotopic resolution of the 22 kDa protein by the benchtop TOF-MS [59]. In all cases, baseline resolution of rhGH from its mono and doubly deamidated forms is possible demonstrating the high power of CE/MS for this type of application. Haselberg et al. used rhGH after heat exposure and prolonged storage showing the possibility to distinguish further protein modifications leading to charge differences [93].

4.6 Glycosylation analysis based on intact proteins

Glycosylation analysis is of great importance in modern biopharmaceutical approaches. CE/MS has been shown to be very useful for the analysis of the glycosylation pattern of intact proteins [37, 93, 97, 100–102]. It can be used to optimize the biotechnological process, to distinguish suppliers, for quality control or for the proof of similarity for follow-on biologics. One of the best characterized glycoproteins and important drug erythropoietin is analyzed routinely by CE. A CE/MS method originally developed by Neusüß, Demelbauer, and Pelzing [103] has been improved and well established in the last years [59, 101]. It can be applied to characterize EPO samples with respect to the glycosylation pattern, easily differentiating EPO alpha and EPO beta [104]. Furthermore, the validated method [105] is able to distinguish many pharmaceutical products and preproduction batches of EPO based on the glycosylation pattern determined by CE/MS and subsequent chemometric approaches applying principal component analysis or cluster analysis [106].

Haselberg et al. used a triple layer coating consisting of polybrene-cyclo dextene-polybrene for the characterization of recombinant human interferon ß-1 [93]. The method enables partial resolution of glycoforms, leading to a distinct pattern that can be used for glycoform profiling.

The analysis of human alpha-acid glycoprotein (AGP) was described by Ongay et al., studying the intact glycosylated protein, the deglycosylated protein, and the released glycans by CE/MS [97]. AGP exhibits a high degree of heterogeneity in the glycan structure due to its five N-glycosylation sites as well as variability of the amino acid backbone leading together with some amino acids variants to about 150 AGP isoforms detected by CE/MS. However, unequivocal carbohydrate structures can only be assigned in conjunction with the analysis of the released glycan. The analysis of the intact protein showed again, that the separation is primarily based on different charges with baseline separation of isoforms differing by one charge (here: sialic acid or amino acid change). Based on differences in the glycosylation pattern, healthy and bladder cancer diseased patients could be distinguished [107].

4.7 Intact proteins in serum

A major obstacle in CE/MS is the small capillary volume which calls for high analyte concentrations in the sample. For this reason, CE/MS is mainly applicable for the characterization and quantification of proteins within pharmaceutical formulations where the protein constitutes a large fraction within the drug and adequate sample amounts can be used. However, high analyte concentrations of biological samples can also be achieved via protein enrichment. For this purpose, specific antibody columns can be used to target the analyte itself, which was used for example to enrich the glycoprotein AGP within human blood plasma with anti-AGP column, where the AGP was retained on the column while matrix and residual proteins were removed [108]. This procedure, however, relies on the availability of an antibody column for the specific analyte protein, which is time and cost consuming to produce. As an alternative, immunodepletion kits for the appropriate sample type can be used which remove the most abundant proteins that interfere with the analysis. This procedure has been used for example for the analysis of hemoglobin-based oxygen carriers like Oxyglobin (a derivative of bovine hemoglobin) in human serum plasma from doping controls by CE/MS [88].

4.8 Protein-drug conjugates

CE/MS was also applied for the characterization of lysozyme–drug conjugates which are used in advanced drug targeting for example in cancer therapy [96]. In such conjugates, several drug molecules are bound to a carrier protein over linker molecules. The attachment of the drug and linker to the carrier protein leads to a distribution of carrier molecule to drug ratios, which needs to be characterized for batch-to-batch consistency and synthesis refinement. In this paper, lysozyme modified by BOC-l-methionine hydroxysuccinimide ester (LZM-BOCmet) was used as carrier protein, which was coupled to the cis-Pt(ethylenediamine) nitrate–chloride (Universal Linkage System; ULS^{TM}). For the drug-conjugate preparations, the platinum-based linker was then coordinated to the kinase inhibitors LY364947, erlotinib, and

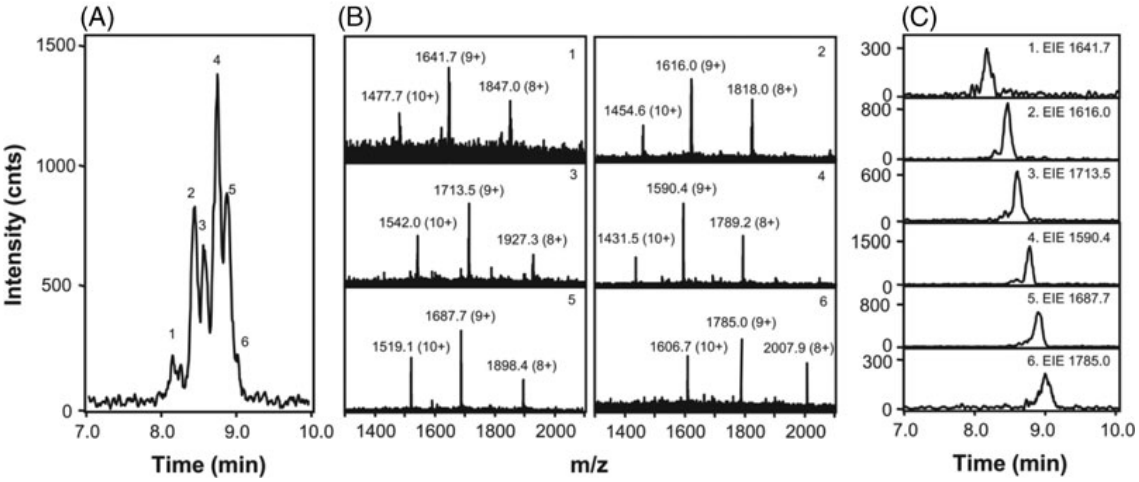

Figure 2. Analysis of an erlotinib-ULS-BOCmet-LZM preparation (drug-conjugate) with CE-TOF-MS, showing BPE (A), mass spectra (B), and EIEs (C). The peaks correspond to (1) $BOCmet_2$-LZM; (2) $BOCmet_1$-LZM; (3) LZM; (4) $(erlotinib\text{-}ULS)_1$-$BOCmet_1$-LZM; (4) $(erlotinib\text{-}ULS)_1$-$BOCmet_2$-LZM; (4) $(erlotinib\text{-}ULS)_2$-$BOCmet_2$-LZM: Reprinted with permission from [96].

Y27632. Figure 2 shows the BPE, mass spectra, and EIEs of an erlotinib-ULS-BOCmet-LZM preparation. Due to charge differences induced by the BOC-l-methionine attachment to lysine residue in the protein, the separation of LZM carrying different number of BOCmet and erlotinib residua into several peaks is possible with CE/MS.

4.9 Metal-protein complexes

CE/MS of intact proteins can also give insights into the stoichiometry of metal-protein complexes. Borges-Alvarez et al. showed that the pH of the BGE is a crucial factor to prevent metal depletion and dissociation of the complexes [58]. The application of 10 mM NH_4Ac at pH 7.3 allowed the detection and differentiation between Cu,Zn-superoxide dismutase (SOD) in its monomeric (Cu,Zn-SOD) and dimeric form (Cu_2,Zn_2-SOD). Moini reported that metal displacement in proteins can also be studied by CE/MS [109]. As proof of concept, the displacement of Co(II) by Cd(II) within the protein N-acyl-L-homoserine-lactonase (AHL) in its monomeric form has been used.

5 Carbohydrates

Next to intact protein and glycopeptide characterization as described before, the detailed characterization of glycosylation based on the released carbohydrates is mandatory since the high variability of glycan structures leads to complex mixtures and several combinations with a certain mass [36, 110].

N-glycans, (N-glycosidic bonded via asparagines) can be easily cleaved from the protein backbone using a glycosidase (e.g. PNGase F). The analyses of N-glycans based on CZE- and CGE-LIF [111–113] are promising and powerful tools in the pharmaceutical industry. The methods present efficient separation of glycan isoforms, short analysis time combined with high sensitivity detection. However, the confirmation and characterization of glycans is time consuming, as CE is not scalable toward a (semi)preparative technique. Therefore, the combination of CE or CE-LIF and mass spectrometry is a fast and helpful tool to identify unknown glycan species [36, 114–116]. The developments in CE/MS for glycan analysis in the years from 2006 to 2011 are summarized in Table 3 and discussed in detail in the following sections.

5.1 Nonderivatized glycans

Generally, the electrophoretic separation and the ESI-ionization process require charged glycans. A common constituent of complex glycans are sialic acids. Due to a negatively charged residue, sialylated glycans can be analyzed after release by CE/MS without any further treatment [37, 117]. One possibility is the analysis in bare fused-silica capillaries with basic background electrolytes. The resulting strong EOF transports the glycans against their mobility from the inlet to the mass detector with high resolving power. Basic BGE systems based on 0.9 M ammonia, 0.1 M 6-aminocaproic acid, and high percentages of methanol (see Table 3) were presented for the characterization of glycans from erythropoietin, fetuin, and AGP [37, 97]. A sheath liquid containing 50% 2-propanol without any further additives was applied. This fast and simple method provides detailed information about type and composition of the released glycans.

Similarly, the analysis of phosphorylated or sulfated glycans is expected to be possible, however, to our knowledge, this has not been shown so far.

Table 3. CE/MS methods and applications for the analysis of glycans in the last years (2006–2011)

Analyte	Coating	BGE	SL	MS	Citation
Fetuin, AGP, and rHuEPO glycans	-	0.1M 6-aminocaproic acid, 0.9 M NH_4Ac in 70% MeOH	IPA/H_2O (50/50 v/v)	QTOF	[37]
AGP glycans	-	0.1 M 6-aminocaproic acid + 0.9 M NH_3 in 40% MeOH	IPA/H_2O (50/50 v/v)	QTOF	[97]
Human plasma N-glycans 2-AA-labeled	-	10 mM HAc /50 mM NH_3 + 50% MeOH 10 mM HAc /50 mM NH_3	IPA/H_2O (50/50 v/v)	QTOF	[123]
Fmoc-labeled glycans	-	50 mM NH_4Ac	MeOH/H_2O (50/50 v/v) + 0.1% v/v FA	Ion trap	[124]
rMAb glycans APTS-labeled	PVA	40 mM 6-aminocaproic acid (pH 4.5, HAc)	IPA/H_2O (50/50 v/v) + 0.2% v/v NH_3	QTOF	[119, 126]
Transferin and fetuin glycans APTS labeled	PVA	IPA/H_2O (20/80 v/v) + 15 mM HAc (pH 4.75)	IPA/H_2O (20/80 v/v) + 15 mM HAc	QTOF	[120]
Human milk oligosaccharides APTS labeled	-	0.3% FA (pH 2.4)	IPA/H_2O (50/50 v/v)	Ion trap	[125]

5.2 Derivatized glycans

Glycans without any ionic group need derivatization prior to CE/MS analysis. Labeling protocols have been recently reviewed [118]. Preferably, well-studied ionic labeling reagents of common CGE-LIF methods are also applied for CE/MS. Widely used derivatization reagents for CGE-LIF that also have been used by CE/MS include the negatively charged 9-aminopyrene-1,3,6-trisulfonic acid (APTS) [119, 120], 8-Aminonaphthalene-1,3,6-trisulfonic acid (ANTS) [121, 122], or 2-aminobenzoic acid (2-AA) [123]. In recent years, CE/MS method development for N-glycans (in the biomedical context) was focused on APTS-labeled glycans. The reason is the widespread use of CGE-LIF systems often applied with a commercially available carbohydrate analysis kit for APTS-labeled N-glycans and the use of an argon-ion laser as a convenient light source [36].

Cationic labeling reagents like the commonly used 2-aminobenzamid (2-AB) in HPLC can also be applied to CE/MS. However, the negative charge of attached sialic acids can form neutral or negatively charged glycans, which should be taken into account during method development. The potential formation of neutral species may constrain the applicability of positively charged labels for sialylated glycans.

5.3 Basic background electrolytes

Derivatized glycans with negatively charged label can be separated in bare fused-silica capillaries and basic BGE systems as described for the sialylated glycans. Ruhaak et al. [123] published a method for 2-AA-labeled glycans in fused-silica capillaries. A separation electrolyte of 10 mM acetic acid, 50 mM ammonia enables a fast separation at normal polarity within 5 min. The addition of 50% methanol can enhance the separation efficiency, since, size and shape of N-glycans get more influence [123]. Nakano et al. [124] published a similar method with 50 mM ammonium acetate and a sheath liquid consisting of 50% methanol and 0.1% of formic acid. However, the presented method is based on an alternative and high-sensitive labeling with 9-fluorenyl chloroformate (Fmoc-Cl). The Fmoc labeling method is described as more rapid compared to other labels due to a simple and direct labeling procedure. Nevertheless, the presented separation of fetuin glycans seems not as efficient as shown for unlabeled fetuin glycans [37].

5.4 Acidic background electrolytes

Methods based on an acidic BGE similar to the advanced CE-LIF method are aimed for CE/MS analysis of APTS-labeled glycans [119, 125]. Hereby, the negatively charged glycans are separated according to their mobility from the cathodic inlet to the anode. Based on a low pH level, the dissociation of capillary silanol groups is reduced and results in a very low electroosmotic flow (EOF). Albrecht et al. [125] used uncoated capillaries for APTS-labeled oligosaccharides in human milk. A BGE of 0.3% formic acid at pH 2.4 suppresses the EOF and enables a separation at reversed polarity. The analysis was performed on an online CE-LIF-MS system in comparison to a routine CE-LIF approach. The standard CE-LIF method was modified for CE-LIF-MS analysis, whereby, for breast milk oligosaccharides 19 peaks in online CE-LIF-MS and 37 peaks in offline CE-LIF were detected. Maxwell et al. [53]

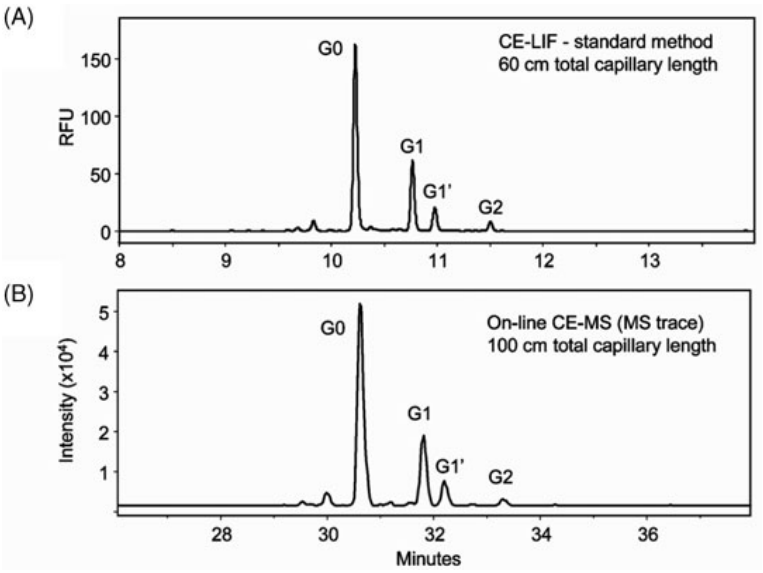

Figure 3. (A) Typical CE-LIF electropherogram of APTS-labeled glycans from an rMAb. (B) CE-MS base peak electropherogram (using the modified method as stated in the text) of glycans from the same rMAb. Reprinted with permission [126].

presented a related method for APTS-labeled glycans in bare fused-silica capillaries. Different BGE compositions of formic or acetic acid and methanol were tested. The best resolution for a glucose ladder standard was achieved by 2% formic acid and 30% methanol. A valuation of this method in respect of separation efficiency is difficult, due to the use of simple glucose unites.

In slightly acidic BGE systems, a neutral coating is used to suppress the EOF. Next to the widespread used polyvinyl alcohol (PVA) coatings, there are several further physically adsorbed and covalent coatings like soluble polyacrylamide and permanent polyacrylamide (PAA), which can be used as neutral EOF suppressing layers as well [25].

Two examples for acidic BGE systems in neutral-coated PVA capillaries for APTS derivatized glycans were presented. Gennaro et al. [119, 126] applied a separation electrolyte of 40 mM 6-aminocaproic acid (pH 4.5 adjusted with acetic acid). The sheath liquid of 50% 2-propanol contains additionally 0.2% ammonia. The CE/MS method was adapted from an existing CE-LIF method. The study showed well-separated glycans, similar to the CE-LIF method; for example, the isomeric glycans G1 and G1′ were clearly separated. Figure 3 presents two electropherograms of isomer separated APTS-labeled glycans from an rMAb for CE-LIF and CE/MS analysis. Szabo et al. [120] used an acidic electrolyte consisting of 15 mM acetate buffer at pH 4.75, the sheath liquid was mixed of 80% BGE and 20% 2-propanol. The method was applied on a very short PVA capillary (30 cm) to optimize several important sample preparation steps for CE-LIF and CE/MS analysis with respect to reaction time and consumption of APTS reagent. The separation efficiency seems to be limited compared to the separation of unlabeled fetuin glycans [37], however, an accurate comparison is difficult.

5.6 Concluding remarks regarding glycan analysis

The analysis of complex glycan mixtures by CE/MS in the pharmaceutical area is still challenging, especially, in terms of high separation efficiency and reproducibility. For glycan analysis, in the (bio)pharmaceutical field, the separation of structural isomers is of high importance. The use of comparable glycan isomer standards, like G1 and G1′ would be reasonable in future reports in order to allow the comparison of separation efficiencies.

At this point, the acidic method based on 6-aminocaproic acid in PVA-coated capillaries [119] seems to be best suited to separate and identify unknown glycans from the standard CE-LIF approach. Unfortunately, the method seems to be difficult to reproduce and not robust in this form (unpublished measurements). Therefore, further developments are necessary for routine glycan analysis by CE/MS. Alternatively, a basic BGE system would be applicable for APTS-labeled glycans, with promising results in terms of structural isomer separation. However, the comparability with common acidic CE-LIF methods is complicated by diverse migration patterns. Furthermore, sensitivity of CE/MS needs to be increased in order to detect minor glycan species, since (i) injection amount is low in CE and (ii) CE/MS reveals a lower sensitivity compared to high-sensitive CE-LIF systems.

6 Validation/quantitation

Method validation is of major importance in the pharmaceutical context with respect to both reliability and quantitation. However, detailed validation is rarely performed as most methods in CE/MS are in an early stage with respect to

routine application and CE/MS is mainly used in research. In general, CE/MS can be used similarly to LC/MS for precise and reliable quantitation [127]. Both CE/MS and LC/MS depend mainly on properties of the electrospray ionization leading to precision in the order of 10%, especially since isotopically labeled internal standards are often not available. Precision in migration time is often low as it depends to a large extent on surface processes (The determination of the EOF and the stability of the coating are of major importance–cp. above). However, using mobilities rather than migration times, the precision is often better than 1% RSD. Dynamic range of CE/MS can be as high as 3–4 orders of magnitude being sufficient for almost any pharmaceutical application [127]. Typical sensitivity achievable in CE/MS is in the upper attomol- to low femtomol-range, depending on the MS used. This corresponds to high ppb range for small molecules injecting a few nanoliters. CE/MS can be robust with respect to several parameters, depending on separation system (BGE, coating) and the interface. The standard sheath-liquid interface with the sprayer on ground is very robust to many parameters, like position of the capillary, sheath liquid composition (high organic content can cause instabilities), flow rate, etc. [128]. A validation of a method for the analysis of intact proteins by CE/MS has been recently performed [105].

7 Concluding remarks

A major limitation of CE (and thus CE/MS) is the lack of concentration sensitivity due to small injection volumes. However, this is not the limiting factor in the context of drug substance or drug formulation analysis. Biological samples are approachable after sample preparation, whereas impurity profiling can be performed straightforward as CE separation is tolerant to overloading. Thus, CE/MS is expected to be much more applied in the pharmaceutical area in the future. This is especially the case where CE/MS shows a beneficial selectivity compared to LC/MS. Various applications of small molecule and peptide analysis show the potential of CE/MS and will certainly lead to a more widespread application of this powerful technique. Intact protein analysis and glycan characterization CE/MS will be a major application of CE/MS in the (bio)pharmaceutical field.

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8 References

[1] Kaltashov, I. A., Bobst, C. E., Abzalimov, R. R., Berkowitz, S. A., Houde, D., *J. Am. Soc. Mass Spectrom.* 2010, *21*, 323–337.

[2] Fang, A. S., Miao, X., Tidswell, P. W., Towle, M. H., Goetzinger, W. K., Kyrandos, J. N., *Mass Spectrom. Rev.* 2008, *27*, 20–34.

[3] Gillespie, T. A., Winger, B. E., *Mass Spectrom. Rev.* 2010, *30*, 479–490.

[4] Drexler, D. M., Reily, M. D., Shipkova, P. A., *Anal. Bioanal. Chem.* 2011, *399*, 2645–2653.

[5] Domann, P. J., Akashi, S., Barbas, C., Huang, L., Lau, W., Legido-Quigley, C., McClean, S., Neusüß, C., Perrett, D., Quaglia, M., Rapp, E., Smallshaw, L., Smith, N. W., Smyth, W. F., Taylor, C. F., *Nat. Biotechnol.* 2010, *28*, 654–655.

[6] Chen, G., Warrack, B. M., Goodenough, A. K., Wei, H., Wang-Iverson, D. B., Tymiak, A. A., *Drug Discov. Today* 2011, *16*, 58–64.

[7] Stokvis, E., Rosing, H., Beijnen, J. H., *Mass Spectrom. Rev.* 2005, *24*, 887–917.

[8] Wuhrer, M., Deelder, A. M., Hokke, C. H., *J. Chromatogr. B* 2005, *825*, 124–133.

[9] Laursen, K., Justesen, U., Rasmussen, M. A., *J. Chromatogr. A* 2011, *1218*, 4340–4348.

[10] Görg, A., Weiss, W., Dunn, M. J., *Proteomics* 2004, *4*, 3665–3685.

[11] Görg, A., Drews, O., Lück, C., Weiland, F., Weiss, W., *Electrophoresis* 2009, *30*, S122–S132.

[12] Scriba, G. K. E., *J. Pharm. Biomed. Anal.* 2011, *55*, 688–701.

[13] Ohnesorge, J., Neusüß, C., Wätzig, H., *Electrophoresis* 2005, *26*, 3973–3987.

[14] Mischak, H., Coon, J. J., Novak, J., Weissinger, E. M., Schanstra, J. P., Dominiczak, A. F., *Mass Spectrom. Rev.* 2009, *28*, 703–724.

[15] Klampfl, C. W., Buchberger, W., *Curr. Anal. Chem.* 2010, *6*, 118–125.

[16] Sniehotta, M., Schiffer, E., Zürbig, P., Novak, J., Mischak, H., *Electrophoresis* 2007, *28*, 1407–1417.

[17] Schmitt-Kopplin, P., Frommberger, M., *Electrophoresis* 2003, *24*, 3837–3867.

[18] Marsh, A., Broderick, M., Altria, K., Power, J., Donegan, S., Clark, B., *Methods Mol. Biol.* 2008, *384*, 205–245.

[19] Altria, K., Marsh, A., Griend, C. S.-v. d., *Electrophoresis* 2006, *27*, 2263–2282.

[20] Klampfl, C. W., *Electrophoresis* 2006, *27*, 3–34.

[21] Hernández-Borges, J., Neusüß, C., Cifuentes, A., Pelzing, M., *Electrophoresis* 2004, *25*, 2257–2281.

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

[23] Staub, A., Guillarme, D., Schappler, J., Veuthey, J.-L., Rudaz, S., *J. Pharm. Biomed. Anal.* 2011, *55*, 810–822.

[24] Horvath, J., Dolník, V., *Electrophoresis* 2001, *22*, 644–655.

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

[26] Cao, F., Luo, Z., Zhou, D., Zeng, R., Wang, Y., *Electrophoresis* 2011, *32*, 1148–1155.

[27] Zhou, D., Tan, L., Xiang, L., Zeng, R., Cao, F., Zhu, X., Wang, Y., *J. Sep. Sci.* 2011, *34*, 1738–1745.

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

[29] Ullsten, S., Zuberovic, A., Bergquist, J., *Methods Mol. Biol.* 2008, *384*, 631–646.

[30] Righetti, P. G., Gelfi, C., Verzola, B., Castelletti, L., *Electrophoresis* 2001, *22*, 603–611.

[31] Robb, C. S., *J. Liq. Chromatogr. Rel. Technol.* 2007, *30*, 729–759.

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

[33] Li, H., d'Anjou, M., *Curr. Opin. Biotechnol.* 2009, *20*, 678–684.

[34] Kawasaki, N., Itoh, S., Hashii, N., Takakura, D., Qin, Y., Huang, X., Yamaguchi, T., *Biol. Pharm. Bull.* 2009, *32*, 796–800.

[35] Kamoda, S., Kakehi, K., *Electrophoresis* 2008, *29*, 3595–3604.

[36] Mechref, Y., Novotny, M. V., *Mass Spectrom. Rev.* 2009, *28*, 207–222.

[37] Balaguer, E., Neusüß, C., *Anal. Chem.* 2006, *78*, 5384–5393.

[38] Quirino, J. P., *Electrophoresis* 2011, *32*, 665–668.

[39] Pantůčková, P., Gebauer, P., Boček, P., Křivánková, L., *Electrophoresis* 2011, *32*, 43–51.

[40] Freudemann, T., von Brocke, A., Bayer, E., *Anal. Chem.* 2001, *73*, 2587–2593.

[41] von Brocke, A., Freudemann, T., Bayer, E., *J. Chromatogr. A* 2003, *991*, 129–141.

[42] Nilsson, C., Birnbaum, S., Nilsson, S., *Electrophoresis* 2011, *32*, 1141–1147.

[43] Chen, C.-J., Wang, C.-W., Her, G.-R., *J. Sep. Sci.* 2011, *34*, 2538–2543.

[44] Klampfl, C. W., *J. Chromatogr. A* 2004, *1044*, 131–144.

[45] Scriba, G. K. E., *J. Chromatogr. A* 2007, *1159*, 28–41.

[46] Maxwell, E. J., Chen, D. D. Y., *Anal. Chim. Acta* 2008, *627*, 25–33.

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

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

[49] Busnel, J.-M., Schoenmaker, B., Ramautar, R., Carrasco-Pancorbo, A., Ratnayake, C., Feitelson, J. S., Chapman, J. D., Deelder, A. M., Mayboroda, O. A., *Anal. Chem.* 2010, *82*, 9476–9483.

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

[51] Liu, C. C., Zhang, J., Dovichi, N. J., *Rapid Commun. Mass Spectrom.* 2005, *19*, 187–192.

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

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

[54] Hommerson, P., Khan, A. M., de Jong, G. J., Somsen, G. W., *J. Chromatogr. A* 2008, *1204*, 197–203.

[55] Hommerson, P., Khan, A. M., Jong, G. J. d., Somsen, G. W., *Electrophoresis* 2007, *28*, 1444–1453.

[56] Hommerson, P., Khan, A. M., Bristow, T., Harrison, M. W., de Jong, G. J., Somsen, G. W., *Rapid Commun. Mass Spectrom.* 2009, *23*, 2878–2884.

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

[58] Borges-Alvarez, M., Benavente, F., Barbosa, J., Sanz-Nebot, V., *Rapid Commun. Mass Spectrom.* 2010, *24*, 1411–1418.

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

[60] Mohr, J., Swart, R., Samonig, M., Böhm, G., Huber, C. G., *Proteomics* 2010, *10*, 1–12.

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

[62] Pantůčková, P., Gebauer, P., Bocek, P., Křivánková, L., *Electrophoresis* 2009, *30*, 203–214.

[63] Desiderio, C., Rossetti, D. V., Iavarone, F., Messana, I., Castagnola, M., *J. Pharm. Biomed. Anal.* 2010, *53*, 1161–1169.

[64] Williams, B. J., Cameron, C. J., Workman, R., Broeckling, C. D., Sumner, L. W., Smith, J. T., *Electrophoresis* 2007, *28*, 1371–1379.

[65] Cavazza, A., Corradini, C., Marini, M., Roda, L. G., Valenti, A., *J. Chromatogr. B* 2011, *879*, 2501–2506.

[66] Mayboroda, O. A., Neususess, C., Pelzing, M., Zurek, G., Derks, R., Meulenbelt, I., Kloppenburg, M., Slagboom, E. P., Deelder, A. M., *J. Chromatogr. A* 2007, *1159*, 149–153.

[67] Shama, N., Bai, S. W., Chung, B. C., Jung, B. H., *J. Chromatogr. B* 2008, *865*, 18–24.

[68] Ramautar, R., Mayboroda, O. A., Derks, R. J. E., van Nieuwkoop, C., van Dissel, J. T., Somsen, G. W., Deelder, A. M., de Jong, G. J., *Electrophoresis* 2008, *29*, 2714–2722.

[69] Wang, S., Yang, P., Zhao, X., *Chromatographia* 2009, *70*, 1479–1484.

[70] Sanchez-Hernandez, L., Luisa Marina, M., Crego, A. L., *J. Chromatogr. A* 2011, *1218*, 4944–4951.

[71] Herrero, M., Ibanez, E., Cifuentes, A., *Electrophoresis* 2008, *29*, 2148–2160.

[72] Soga, T., *Methods Mol. Biol.* 2007, *358*, 129–137.

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

[74] Ramautar, R., Somsen, G. W., de Jong, G. J., *Electrophoresis* 2009, *30*, 276–291.

[75] Mischak, H., Schanstra, J. P., *Proteomics Clin. Appl.* 2011, *5*, 9–23.

[76] Ahmed, F. E., *J. Chromatogr. B* 2009, *877*, 1963–1981.

[77] Catala-Clariana, S., Benavente, F., Gimenez, E., Barbosa, J., Sanz-Nebot, V., *Anal. Chim. Acta* 2010, *683*, 119–125.

[78] Staňová, A., Marák, J., Rezeli, M., Páger, C., Kilár, F., Kaniansky, D., *J. Chromatogr. A* 2011, *1218*, 8701–8707.

[79] Taichrib, A., Scriba, G., Neusüß, C., *Anal. Bioanal. Chem.* 2011, *401*, 1365–1375.

[80] Pelzing, M., Neusüß, C., *Electrophoresis* 2005, *26*, 2717–2728.

[81] Znaleziona, J., Maier, V., Ranc, V., Sevcik, J., *J. Sep. Sci.* 2011, *34*, 1167–1173.

[82] Elhamili, A., Samuelsson, J., Bergquist, J., Wetterhall, M., *Electrophoresis* 2011, *32*, 647–658.

[83] Elhamili, A., Bergquist, J., *Electrophoresis* 2011, *32*, 1778–1785.

[84] Scriba, G., *Electrophoresis* 2003, *24*, 2409–2421.

[85] Simó, C., García-Cañas, V., Cifuentes, A., *Electrophoresis* 2010, *31*, 1442–1456.

[86] Sungthong, B., Iványi, R., Bunz, S.-C., Neusüß, C., Scriba, G. K. E., *Electrophoresis* 2010, *31*, 1498–1505.

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

[88] Staub, A., Rudaz, S., Saugy, M., Veuthey, J.-L., Schappler, J., *Electrophoresis* 2010, *31*, 1241–1247.

[89] Staub, A., Giraud, S., Saugy, M., Rudaz, S., Veuthey, J.-L., Schappler, J., *Electrophoresis* 2010, *31*, 388–395.

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

[91] Dolník, V., *Electrophoresis* 2008, *29*, 143–156.

[92] Graf, M., García, R. G., Wätzig, H., *Electrophoresis* 2005, *26*, 2409–2417.

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

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

[95] Catai, J. R., Torano, J. S., de Jong, G. J., Somsen, G. W., *Analyst* 2007, *132*, 75–81.

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

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

[98] Gonzalez, N., Elvira, C., San Roman, J., Cifuentes, A., *J. Chromatogr. A* 2003, *1012*, 95–101.

[99] Catai, J. R., Sastre Torano, J., Jongen, P. M. J. M., de Jong, G. J., Somsen, G. W., *J. Chromatogr. B* 2007, *852*, 160–166.

[100] Sanz-Nebot, V., Balaguer, E., Benavente, F., Neusüß, C., Barbosa, J., *Electrophoresis* 2007, *28*, 1949–1957.

[101] Balaguer, E., Demelbauer, U., Pelzing, M., Sanz-Nebot, V., Barbosa, J., Neusüß, C., *Electrophoresis* 2006, *27*, 2638–2650.

[102] Thakur, D., Rejtar, T., Karger, B. L., Washburn, N. J., Bosques, C. J., Gunay, N. S., Shriver, Z., Venkataraman, G., *Anal. Chem.* 2009, *81*, 8900–8907.

[103] Neusüß, C., Demelbauer, U., Pelzing, M., *Electrophoresis* 2005, *26*, 1442–1450.

[104] Balaguer, E., Neusüß, C., *Chromatographia* 2006, *64*, 351–357.

[105] Taichrib, A., Pioch, M., Neusüß, C., *Electrophoresis* 2012, *33*, doi:10.1002/elps.201100620

[106] Taichrib, A., Pioch, M., Neusüß, C., *Anal. Bioanal. Chem.*, 1-9, doi:10.1007/s00216-012-5924-8

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

[108] Ongay, S., Neusüß, C., Vaas, S., Díez-Masa, J. C., Frutos, M. d., *Electrophoresis* 2010, *31*, 1796–1804.

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

[110] Nakano, M., Kakehi, K., Taniguchi, N., Kondo, A., in: Volpi, N. (Ed.), *Capillary Electrophoresis of Carbohydrates*, Humana Press Inc., New York 2011, pp. 205–235.

[111] Schwarzer, J., Rapp, E., Reichl, U., *Electrophoresis* 2008, *29*, 4203–4214.

[112] Khandurina, J., Blum, D. L., Stege, J. T., Guttman, A., *Electrophoresis* 2004, *25*, 2326–2331.

[113] Kamoda, S., Ishikawa, R., Kakehi, K., *J. Chromatogr. A* 2006, *1133*, 332–339.

[114] Novotny, M. V., Soini, H. A., Mechref, Y., *J. Chromatogr. B* 2008, *866*, 26–47.

[115] Campa, C., Coslovi, A., Flamigni, A., Rossi, M., *Electrophoresis* 2006, *27*, 2027–2050.

[116] Amon, S., Zamfir, A. D., Rizzi, A., *Electrophoresis* 2008, *29*, 2485–2507.

[117] Gennaro, L. A., Delaney, J., Vouros, P., Harvey, D. J., Domon, B., *Rapid Commun. Mass Spectrom.* 2002, *16*, 192–200.

[118] Ruhaak, L., Zauner, G., Huhn, C., Bruggink, C., Deelder, A., Wuhrer, M., *Anal. Bioanal. Chem.* 2010, *397*, 3457–3481.

[119] Gennaro, L. A., Salas-Solano, O., *Anal. Chem.* 2008, *80*, 3838–3845.

[120] Szabo, Z., Guttman, A., Rejtar, T., Karger, B. L., *Electrophoresis* 2010, *31*, 1389–1395.

[121] Zamfir, A., Peter-Katalinic, J., *Electrophoresis* 2004, *25*, 1949–1963.

[122] Larsson, M., Sundberg, R., Folestad, S., *J. Chromatogr. A* 2001, *934*, 75–85.

[123] Ruhaak, L. R., Huhn, C., Waterreus, W.-J., de Boer, A. R., Neusüß, C., Hokke, C. H., Deelder, A. M., Wuhrer, M., *Anal. Chem.* 2008, *80*, 6119–6126.

[124] Nakano, M., Higo, D., Arai, E., Nakagawa, T., Kakehi, K., Taniguchi, N., Kondo, A., *Glycobiology* 2009, *19*, 135–143.

[125] Albrecht, S., Schols, H. A., Heuvel, E. G. H. M. v. d., Voragen, A. G. J., Gruppen, H., *Electrophoresis* 2010, *31*, 1264–1273.

[126] Liu, Y., Salas-Solano, O., Gennaro, L. A., *Anal. Chem.* 2009, *81*, 6823–6829.

[127] Ohnesorge, J., de Griend, C. S. V., Watzig, H., *Electrophoresis* 2005, *26*, 2360–2375.

[128] Geiser, L., Rudaz, S., Veuthey, J.-L., *Electrophoresis* 2003, *24*, 3049–3056.

[129] Sanchez-Hernandez, L., Garcia-Ruiz, C., Crego, A. L., Luisa Marina, M., *J. Pharm. Biomed. Anal.* 2010, *53*, 1217–1223.

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

Manuscript 2

Analysis of native and APTS-labeled N-glycans by capillary electrophoresis/time-of-flight-mass spectrometry

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Analytical and Bioanalytical Chemistry, 405 (25): 8277-8284 (2013)

The manuscript presents a robust CE-MS method for the analysis of APTS-labeled glycans as well as unlabeled charged glycans based on an alkaline BGE. The method was validated and applied for glycan identification of pharmaceutical products. The ability to determine labeling and ionization efficiency of APTS-labeled samples is rare in this field.

Candidate's work:	Implementation of an alkaline CE-TOF-MS system for the analysis of APTS-labeled and native glycans, sample preparation, method validation, determination of label and ionization efficiency, application to pharmaceutical products, data processing and interpretation, glycan identification, manuscript preparation

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RESEARCH PAPER

Analysis of native and APTS-labeled N-glycans by capillary electrophoresis/time-of-flight mass spectrometry

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Abstract The glycosylation of proteins is of particular interest in biopharmaceutical applications. The detailed characterization of glycosylation based on the released carbohydrates is mandatory since the protein stability, folding, and efficacy are strongly dependent on the structural diversity inherent in the glycan moieties of a glycoprotein. For glycan pattern analysis, capillary electrophoresis with laser-induced fluorescence using 8-aminopyrene-1,3,6-trisulfonic acid (APTS)-labeled glycans is used frequently. In this paper, a robust capillary electrophoresis–mass spectroscopy method both for the analysis of APTS-labeled glycans and unlabeled charged glycans is presented. The background electrolyte consists of 0.7 M ammonia and 0.1 M ε-aminocaproic acid in water/methanol 30:70 (*v*/*v*). High separation efficiency including separation of structural isomers was obtained. The method was validated in terms of reproducibility and linearity. Submicromolar sensitivity is achieved with linearity up to 24 μM. The ability to analyze APTS-labeled, as well as unlabeled, charged glycans enables the determination of labeling and ionization efficiency: APTS-labeled glycans show a factor of three better ionization efficiency compared to non-labeled native glycans. The presented method is applied to the analysis of pharmaceutical products. Furthermore, the system can be applied to the analysis of 2-ANSA-labeled glycans, though separation efficiency is limited.

Keywords APTS · 2-ANSA · CE-MS · Glycan · Glycosylation

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Abbreviations

AGP	α_1-Acid glycoprotein
2-ANSA	2-Aminonaphthylsulfonic acid
APTS	8-Aminopyrene-1,3,6-trisulfonic acid
EACA	8-Aminocaproic acid
CFG	Consortium for Functional Glycomics
CSE	Capillary sieving electrophoresis
SL	Sheath liquid

Introduction

The development of protein-based pharmaceutical products has increased in recent decades, due to their high potential to selectively treat diseases. Many pharmaceutically relevant proteins including antibodies are glycosylated. The study of glycosylation can be performed on different principles. Next to intact protein and glycopeptide characterization, the detailed characterization of glycosylation based on released carbohydrates is mandatory since the protein stability, folding, and efficacy are strongly dependent on specific glycosylation [1–6]. The analysis of N-glycans based on capillary electrophoresis with laser-induced fluorescence (CE-LIF) is widespread and a fast tool in the pharmaceutical industry [7–9]. In this context, derivatization is mandatory for glycans with respect to separation (permanent charge) and detection (fluorescence) [10]. The most abundant derivatization reactions in this field are based on a reductive amination using the reducing glycan termini; thereof, 8-aminopyrene-1,3,6-trisulfonic acid (APTS) is one of the most popular labeling agents [11–13]. Furthermore, commercially available analysis kits promote widespread application. CE-LIF for APTS-labeled glycans often uses pseudogels, which are high-viscosity compounds that achieve sieving effects (i.e., separation due to size) [7, 14]. These capillary sieving electrophoresis (CSE—term is used

according to IUPAC nomenclature [15]) methods with LIF detection provide high resolving power, i.e., separation of structural isoforms and short analysis time combined with highly sensitive detection. However, since the direct confirmation and characterization of glycans is not possible by LIF detection, an alternative detection technique is required. The combination of CE and mass spectrometry is a powerful tool to identify unknown glycan species [13, 16–20]. However, the common CSE-LIF methods are not compatible with mass spectroscopy (MS) detection, since applied pseudogels and electrolytes as phosphates are not volatile. Few CE-MS methods using APTS-labeled glycans have been presented [11, 20–23]. However, the separation efficiencies are either low or difficult to compare as different glycans have been analyzed. The acidic CE-MS system by Gennaro and Salas-Solano and Liu et al. [11, 22] presents high separation efficiency (i.e., G1F and G1F′ can be separated, see structure in Fig. 1) comparable to the here-proposed method. The presented CE-MS methods use acidic background electrolyte (BGE) systems, e.g., based on formic acid [21, 23] or ε-aminocaproic acid (EACA) [11, 20, 22]. Acidic CE-MS systems with negligible electroosmotic flow (EOF) might be sensitive to pressure influences (i.e., siphoning effects, nebulizer gas flow, etc.) and bear the risk of air or SL intake or creation of a moving ion boundary from outlet to inlet. CE-MS systems creating an EOF in MS direction as basic BGE systems can prevent these effects. However, CE-MS methods using basic BGEs have been developed for the analysis of native glycans [24–26].

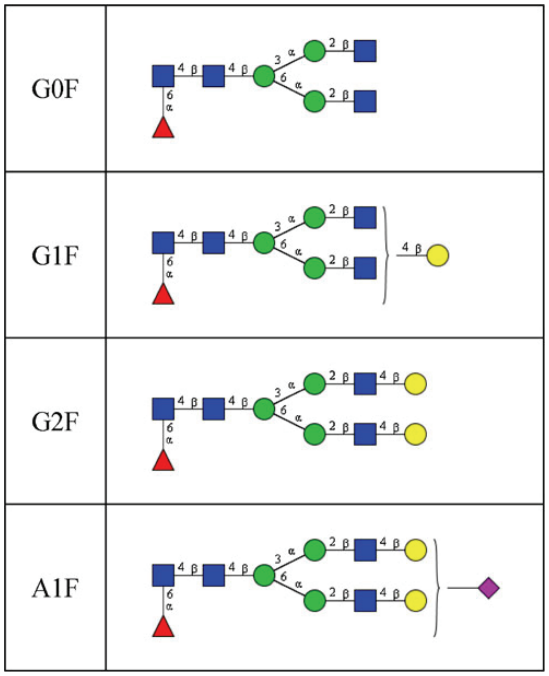

Fig. 1 Structures of all glycan standards used in this work. The glycan structures are displayed according to the CFG nomenclature: *blue squares*, *N*-acetylgalactosamine; *green circles*, mannose; *yellow circles*, galactose; *red triangles*, fucose; *violet diamond*, *N*-acetylneuraminic acid

Here, a robust CE-MS method based on a basic electrolyte with cathodic detection for APTS-labeled glycans as well as charged native glycans was developed and validated. The basic BGE creates a strong EOF towards the MS, thus, carries the negatively charged native glycans (bearing sialic acids, sulfonic acids, or phosphates) as well as APTS-labeled glycans to the MS, resulting in high resolution power. As indicator for acceptable separation efficiency, the resolution of the two isomeric glycans of G1F is chosen, as these glycans are often abundant in antibodies. G1F-glycans are asialo-, biantennary complex-type N-glycans with one galactose attached to either end of the antennae (Fig. 1). Additionally to APTS-labeled glycans, the 2-ANSA label was applied on this CE-MS method. As shown for 5-ANSA, this type of label is applicable to HPLC, CE, and—in contrast to APTS—also to matrix-assisted laser desorption/ionization (MALDI) mass spectrometry [27, 28]. However to our knowledge, no CE-MS method has been presented.

Materials and methods

All solutions were prepared using ultrapure water (18 MΩ cm at 25 °C, SG Ultra Clear UV from Siemens Water Technologies, USA). 2-Propanol, methanol (each LC-MS grade), acetic acid (p.a.), formic acid (p.a.), sodium hydroxide (p.a.), and aqueous ammonia (30 % p.a.) were obtained from Carl Roth GmbH und Co. KG (Karlsruhe, Germany). Ammonium acetate (p.a.), ammonium carbonate (p.a.), EACA, sodium cyanoborohydride ($NaCNBH_3$) in 1.0 M tetrahydrofuran, and citric acid monohydrate (p.a.) were obtained from Sigma-Aldrich (Steinheim, Germany). “ES Tuning mix” solution was obtained from Agilent Technologies (Waldbronn, Germany). Native glycan standards, APTS-labeled, and 2-ANSA-labeled IgG glycans were obtained from Ludger (Abingdon, UK). APTS-labeled glycans of pharmaceutical products were provided by Merck Serono (Rome, Italy). APTS-labeled glycans from bovine α1-acid glycoprotein (AGP) were obtained from Solvias (Basel, Switzerland). APTS-labeled A1F standard was partially labeled as follows: 5 μg of glycan standard was labeled with 1.5 μL APTS solution (20 mM APTS in 0.6 M citric acid), 1.5 μL 0.6 M citric acid, and 1.0 μL 1.0 M $NaCNBH_3$ in tetrahydrofuran for 1 h at 37 °C. After incubation, 21 μL water was added resulting in a final concentration of 96 μM.

CE

CE experiments were performed in fused silica capillaries of 90, 60, or 43 cm length (Polymicro Technologies, AZ, USA) at +30 kV (inlet) on a CE 7100 instrument (Agilent, Waldbronn, Germany). Background electrolytes were based on ammonia and EACA, ammonium acetate or ammonium carbonate, each containing a high percentage of methanol as described in the text. After optimization, the final BGE contained 0.1 M EACA and 0.7 M ammonia in 30:70 water/methanol with an apparent pH of 10. The capillary was flushed with methanol, 1 M sodium hydroxide, and water before the first use or after storage. The sample was injected hydrodynamically by the application of a pressure of usually 50 mbar for 12 s. The injection was followed by dipping the capillary into a water vial in order to prevent contamination of the BGE. For CZE-MS coupling, a coaxial sheath–liquid interface was used (Agilent Technologies). Sheath liquid of 2-propanol/water 1:1 (*v*/*v*) is supplied by syringe pump at a flow rate of 4 μL/min.

MS

MS experiments were performed on a Q-TOF instrument (microTOFQ, Bruker Daltronik, Bremen, Germany). Electrospray ionization was used in negative ion mode applying 4.0 kV on the MS inlet (sprayer on ground). The flow rate of dry gas (nitrogen) was 4.0 L/min at a temperature of 170 °C. The nebulizer gas pressure was set to 0.2 bar. Spectra were acquired in a mass range of *m*/*z* 300–2,500 at a rate of 1 Hz (summation of 5,000 single spectra). An external calibration was performed by infusing tuning mix solution at 4 μL/min every morning. As post-processing, the data were internally calibrated based on observed sodium–EACA clusters. The identification of glycans was based on the accurate mass of doubly and triply charged glycans (mass deviation <5 ppm). Data analysis was performed using DataAnalysis Version 4.0 from Bruker Daltronik (Bremen, Germany). The identification could be verified by comparison of the isotopic distribution, i.e., overlaying the simulated mass spectra with the measured mass spectra. All glycan structures are displayed using the simplified Consortium for Functional Glycomics (CFG) nomenclature: square, *N*-acetylhexosamine; circle, hexose; trangle, fucose; diamond, sialic acid.

Results and discussion

Method development

Starting point of the development was a method for the analysis of native glycans using a BGE of 0.1 M EACA and 0.9 M ammonia with methanol in a ratio of 30:70 [24]. Since the application of this method to APTS-labeled glycans resulted in broad peaks and reduced separation, an appropriate method was developed. The BGE contained ammonia, EACA, and methanol at varying concentration. The decrease of ammonia concentration in BGE from 0.9 to 0.7 M improves the resolution of the APTS-labeled glycans, while peaks got sharper and the intensities increase. Further reduction of ammonia results in longer analysis time, with hardly any improvement in separation efficiency. In a range of 50–70 % methanol, a higher percentage of methanol enhances the peak resolution and intensities, while increasing the analysis time. In the proposed method, a concentration of 70 % of methanol was chosen in order to separate isomeric glycans. Lower methanol content can be applied if shorter analysis time is required. Reduction to 50 % methanol shortens the analysis by a factor of 1.5.

Alternative background electrolytes as 100 and 50 mM ammonium acetate and 50 mM ammonium carbonate each in 50 % methanol/water (*v*/*v*) were tested. However, no acceptable separation (i.e., low resolving power and broad peaks) could be achieved. Finally, the separation was performed using a BGE of 0.7 M ammonia and 0.1 M EACA in 70 % aqueous methanol in bare fused silica capillaries with an apparent pH of 10 (measured with water-based calibration of pH electrode).

The presented method offers the possibility to analyze APTS-labeled in parallel with charged, unlabeled glycans as sialylated glycans (negatively charged in alkaline BGE). Figure 2 shows the base peak electropherogram of α1-acid glycoprotein glycans (AGP, bovine), which were partially labeled by APTS. The charged native glycans (22–31 min) are clearly separated from the APTS-labeled glycans (31–45 min). The main peaks are biantennary N-glycans of complex type with different amounts of sialic acids as annotated in Fig. 2 being detected both as labeled and unlabeled species. The non-labeled biantennary glycan without sialic acid is uncharged, thus migrates with the EOF and was not detected by ESI-MS. This example presents nicely the ability of the new method for parallel separation of charged native and APTS-labeled glycans.

Separation efficiency

The CSE-LIF methods provide high separation efficiency, which is important for glycan analysis in the biopharmaceutical sector. Therefore, an important method criterion for CE-MS is high separation power, e.g., the ability to separate structural isomeric glycans similar to CSE-LIF. The separation of the two isoforms of G1F, as abundant structural isomers in protein-based biopharmaceuticals, was examined as marker for efficient structural isomer separation. The isomers were separated due to their different effective size (charge due to dissociation of APTS is expected to be

identical). The 1,3-linked isoform showed shorter migration time than the 1,6-linked glycan. A resolution of 1.2 was obtained for G1F of a recombinant fusion protein using the proposed CE-MS system, though in CSE-LIF a resolution of 1.8 could be achieved using the method of Ruhaak et al. [29] (data not shown). The differences in CE-MS and CE-LIF were probably caused by sieving effects of the pseudogel electrolyte in CSE-LIF as well as different BGE compositions (e.g., pH, solvation of the analytes, viscosity).

The overall separation efficiency is demonstrated for a complex glycan mixture of a therapeutic antibody. The 15 glycans are baseline separated (compare Fig. 5, depicted later), including further pairs of structural isomers beside G1F.

Validation

Reproducibility experiments based on APTS-labeled glycans from a recombinant fusion protein were conducted in 12 runs over 5 months. The measurements were analyzed in terms of variance in migration time, relative peak ratio, and separation efficiency based on the four main glycan species G0F, G1F, G1F′, and G2F (see structures Fig. 1). Different capillaries with same capillary lengths (90 cm) were applied. The relative standard deviations of migration time, relative peak ratio (intensity) for the four main glycans (G0F, G1F/G1F′, and G2F) and the resolution based on the FWHM of G1F/G1F′ were determined, as listed in Table 1. The three parameters show an acceptable precision for the characterization of glycans.

The linearity was determined using APTS-labeled A1F in a concentration range of 1.5–48 μM. A1F is a monosialylated, core-fucosylated biantennary complex-type N-glycan (Fig. 1). This naturally charged glycan was chosen since the actual concentration of APTS-labeled glycan (i.e., corrected for labeling efficiency) could be determined using the approach described in the following paragraph. A concentration of 48 μM APTS-labeled A1F in stock solution was calculated. Based on this stock solution, all calibration standards were diluted. Instead of a 90-cm capillary length as used for separation, here, a length of 43 cm is used, since there is no need of high separation efficiency in this purpose and analysis time can be reduced. However, a comparison of peak width and intensity in 43 to 90-cm-long capillaries was performed to ensure the applicability of 43 cm capillary length in this context. Furthermore, about the same amount of analyte was injected (10 nL of sample, injection 6 s instead of 12 s at 50 mbar) to ease the transfer to standard separation conditions. Each concentration was analyzed twice; the calibration plot is displayed in Fig. 3.

The calibration regression factor of 0.9988 was calculated in a range of 1.5–24 μM. At a level of 48 μM, the calibration curve is no longer linear, as shown in Fig. 3. A limit of detection of 0.2 μM (1.9 fmol injected) and a limit of quantitation of 0.7 μM (6.7 fmol injected) was calculated based on signal-to-noise-ratio of 3 and 10, respectively. Thereof, a total amount of about 1 pmol of glycan arises from the required sample amount of about 5 μL in CE. The dynamic range of almost 2 orders of magnitude is appropriate for the application to glycan identification of biopharmaceutical products as concentrations are frequently in the nanomolar to low micromolar range and quantitation is usually not performed by CE-MS but rather by CE-LIF.

Ionization and labeling efficiency of APTS-labeled glycans

The presented method enabled the determination of labeling efficiency for APTS-labeled sialylated glycans, since the unlabeled glycans were detectable in parallel. Also, the different ionization behavior of APTS-labeled to unlabeled glycans could be determined. Therefore, a native A1F glycan sample was measured first. Afterwards, the same sample solution was partially APTS-labeled (at 37 °C for 1 h, see

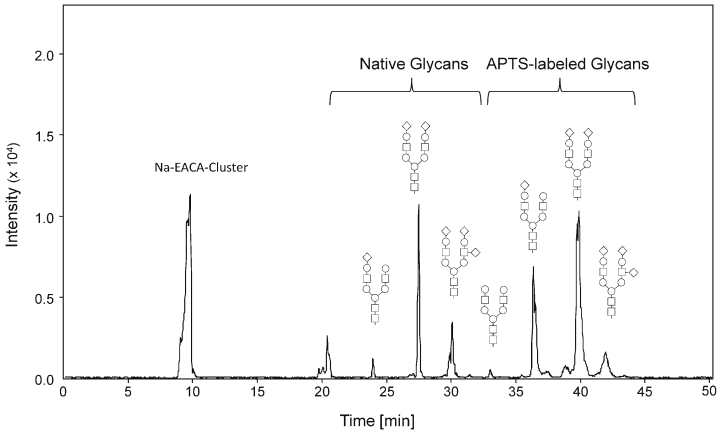

Fig. 2 Base peak electropherogram of APTS-labeled glycans from bovine AGP separated in fused silica capillary of 86 cm length applying the proposed basic BGE system (0.1 M EACA and 0.7 M ammonia in 30:70 water/methanol). The main glycans are annotated: *squares*, *N*-acetylhexosamine; *circles*, hexose; *triangles*, fucose; *diamonds*, sialic acid

Table 1 Precision based on the main APTS-labeled glycans (G0F, G1F, and G2F) of a recombinant fusion protein. Twelve analyses over 5 months were performed according to the proposed method (see Fig. 2) using capillaries of 90 cm length

	Mean	RSD [%]
Relative peak ratios		
G0F/G1F	1.4	5.9
G1F/G1F'	4.3	7.9
G1F/G2F	2.7	11.2
G0F/G2F	1.9	8.3
Migration time		
G0F	1,575 s	3.1
G1F	1,629 s	3.8
G1F'	1,651 s	4.0
G2F	1,704 s	2.6
Resolution		
G1F/G1F'	1.2	15.4

"Materials and methods") to obtain a solution with native and labeled glycans. The unlabeled amount of A1F in the labeled sample was quantified based on the first analyzed native sample. Assuming the rest to be labeled, the ionization efficiency could be calculated. Figure 4 shows the electropherograms of the analyzed native and labeled A1F sample. About 4 % of the A1F standard was found to be desialylated (A1F → A0F) during the labeling process (peak 3). Two percent desialylated A1F was observed at the peak of A1F obviously related to a desialylation in the mass spectrometer. The labeled peak area was calculated by summing up all labeled peak areas, sialylated and desialylated A1F, assuming that the error caused by differences in ionization efficiency is negligible. The native A1F sample and the APTS-labeled solution of A1F were analyzed twice within some weeks. Table 2 shows the areas of unlabeled, expected labeled (assuming the same response as unlabeled), and detected labeled peak area related each to the unlabeled native A1F sample. The ionization of the APTS-labeled glycan (here, A1F) was a factor of 2.9 enhanced compared to the non-labeled glycan.

The calculated labeling efficiency was about 44 % for the A1F standard using the described labeling procedure. For pharmaceutical products, the labeling procedure should be adjusted dependent on the containing glycans. Highly sialylated glycan mixtures are usually treated carefully (at 37 °C) to avoid loss of sialic acids [12]. Hence, for provided pharmaceutical products with sialylated glycans, a labeling efficiency of around 50 % was determined. For glycan mixtures containing no sialic acids, the labeling reaction time could be expanded and the reaction temperature decreased (60 °C); thus, a labeling efficiency of 80–90 % was determined.

Application to pharmaceutical products

The method was successfully applied to identify and characterize the APTS-labeled glycans of three different pharmaceutical products: a therapeutic antibody, a recombinant fusion protein, and a cytokine-based product. The identification of glycans was based on accurate mass of doubly and triply charged glycans with an acceptable mass deviation (<5 ppm). In this way, TOF/MS enabled the calculation of possible glycan compositions; however, neither the differentiation between isobars as different hexoses or hexosamines nor linkage determination was possible. MS/MS experiments might allow the determination of glycan sequence by cleavage of glycosidic bonds. However, for APTS-labeled glycans, cross-ring cleavages are rare; hence, information about linkage position are missing [19]. For all three applied

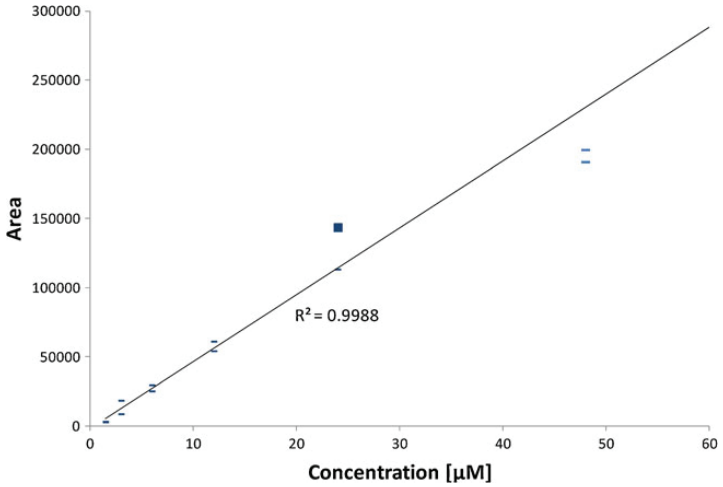

Fig. 3 Calibration plot of APTS-labeled A1F in a range of 1.5–48 µM. The regression curve is calculated up to 24 µM. The second value of 24 µM (*filled square*) is defined as outlier

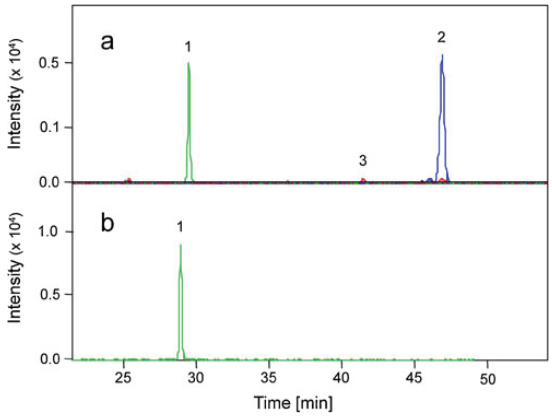

Fig. 4 Extracted ion electropherogram (EIE) of unlabeled A1F (*1*), APTS-A1F (*2*), and desialylated APTS-A1F (*3*) (using the doubly, triply, and fourfold charged glycans). a EIE of an APTS-labeled A1F sample; b EIE of unlabeled A1F sample (48 μM A1F each). Experiments were performed applying the proposed CE-MS method using a capillary length of 90 cm (BGE as given in Fig. 2)

biopharmaceutical products, the released APTS-labeled glycan pool was analyzed. Thirteen glycans were derived from the recombinant fusion protein and seven glycans from the cytokine product. The applied therapeutic antibody bears the most complex glycan pool with 15 different glycan structures including three structural isomer pairs. Therefore, as an example, the analysis of the antibody-derived glycans is presented in Fig. 5. The overlaid, extracted ion electropherograms of all characterized glycans demonstrates the high resolving power of the proposed method for complex glycan compositions of biopharmaceutical samples. The APTS-labeled glycan pool was in parallel analyzed by CSE-LIF (not shown). The migration order was generally reversed due to the cathodic instead of anodic detection. All glycans determined by CSE-LIF (method by Ruhaak et al. [29]) were found also by CE-MS as annotated in Fig. 5. Three so far not identified glycans could be characterized by CE-MS.

Table 2 The relative peak areas of unlabeled glycan standard in two labeled samples are normalized to the separately analyzed native sample (line 1). Assuming the rest of A1F labeled, the relative labeled peak areas are calculated (line 2). The detected peak area of APTS-labeled A1F related to the native sample (line 3). The resulting ionization enhancement factor for APTS-labeled glycan is shown in line 4. Experiments were performed applying the proposed CE-MS method using a capillary length of 90 cm (BGE as given in Fig. 2)

	Labeled sample 1	Labeled sample 2
Area of unlabeledA1F	54	59
Expected area of labeled A1F	46	41
Detected area of labeled A1F	134	121
Ionization enhancement factor	2.9	2.9

Application to 2-ANSA-labeled samples

As an alternative labeling reagent, 2-ANSA was tested for IgG glycans. As shown for 5-ANSA, this type of label is applicable to HPLC, CE-LIF, and—in contrast to APTS—also to MALDI mass spectrometry [27]. Comparing 2-ANSA and APTS, the proposed CE-MS method with normal polarity was employed on an IgG glycan sample in bare fused silica capillaries of 60 cm length. The capillary length was reduced to decrease the analysis time, while the differences in separation efficiency were still determinable. The analysis of APTS- and 2-ANSA-labeled glycans applying the here-presented CE-MS method is shown in Fig. 6. The comparison of these two labels demonstrates the high potential of APTS in terms of separation efficiency. The ion electropherograms of G0F, G1F, and G2F glycans of IgG were extracted to ease the comparison. Anticipated reduced separation efficiency was demonstrated for ANSA-labeled glycans. Since, the smaller number of sulfonic acids of 2-ANSA decreases the mobility against the EOF and, hence, the resulting resolving power due to a shorter migration time. Modifying the BGE in a way that the EOF is reduced (increase of methanol (90 %) and decrease of ammonia to 0.23 and 0.35 M in the BGE) did not improve the separation to an extent as obtained for APTS-labeled glycans. Alternatively, 2-ANSA-labeled samples might be

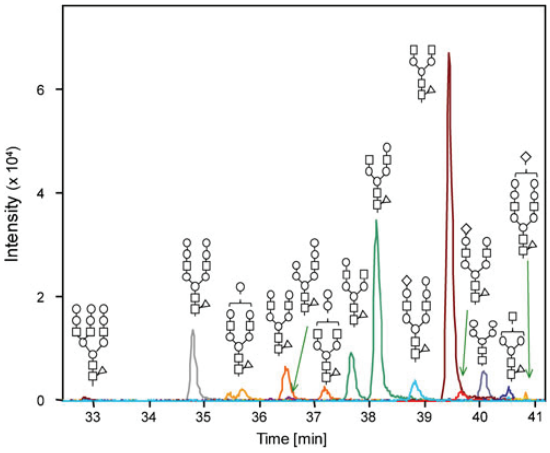

Fig. 5 EIE of the main glycans of a therapeutic antibody (using the doubly, triply, and fourfold charged glycans). Separation was performed with the proposed BGE system (as given in Fig. 2) using a fused silica capillary (90 cm). *Squares*, *N*-acetylhexosamine; *circles*, hexose; *triangles*, fucose; *diamonds*, sialic acid

separated in acidic background electrolytes. Using an acidic separation buffer in a CSE-LIF approach, Briggs et al. [27] obtained an increased separation efficiency of glycans modified with single sulfonated label (5-ANSA) compared to the application of trisulfonated APTS label.

Here, the glycan intensities were increased using the 2-ANSA-label, explicable by higher hydrophobicity of 2-ANSA compared to APTS increasing the ionization efficiency, as described by Walker for different cationic glycan labels [30].

Conclusion

This paper presents a novel CE-TOF-MS method with basic background electrolyte for the analysis of APTS-labeled glycans. The basic background electrolyte enabled a robust CE-MS application due to its strong EOF, minimizing pressure and flow effects at the capillary outlet. Good separation efficiency, i.e., separation of G1F/G1F′ was obtained for biopharmaceutical samples. The sensitivity in the low femtomole range (picomole of sample required) is appropriate for this application. The identification of unknown glycans for different types of biopharmaceutically relevant proteins supports this finding. A detailed and systematic study on the identification of glycans analyzed by different CSE-LIF methods using this proposed as well as an acidic CE-MS method will be following soon.

The possibility to analyze APTS-labeled and charged, native glycans simultaneously with high separation efficiency is unique in this field. The proposed method enables the determination of labeling and ionization efficiency. Although the APTS label does not introduce hydrophobicity, the ionization efficiency is a factor 3 higher than for native glycans. In comparison to APTS-labeled glycans, the separation efficiency for 2-ANSA-labeled glycans applying the presented method is reduced, while the ionization efficiency was increased. Nevertheless, the proposed method is applicable to glycans derivatized by other acidic labels such as 2-ANSA enabling a complementary analysis in addition to, e.g., MALDI-TOF-MS.

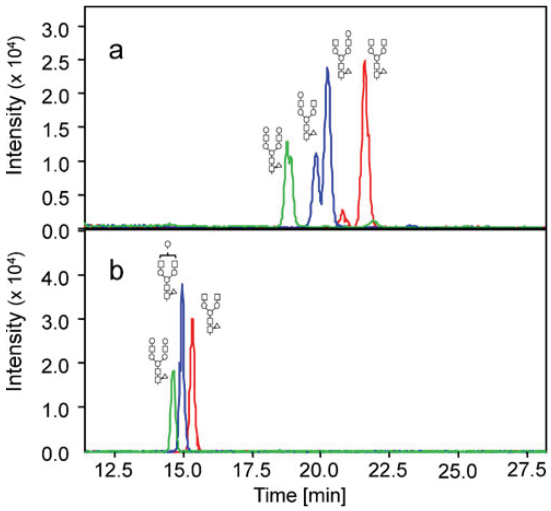

Fig. 6 EIE of G0F, G1F, and G2F (using the doubly, triply, and fourfold charged glycans). a EIE of APTS-labeled IgG glycan sample; b EIE of 2-ANSA-labeled IgG glycan sample. Separation was performed applying the proposed BGE system (see Fig. 2) using a fused silica capillary of 60 cm length. *Squares*, *N*-acetylhexosamine; *circles*, hexose; *triangles*, fucose; *diamonds*, sialic acid

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Conflict of interest The authors declare no conflict of interest.

References

1. Sola RJ, Griebenow K (2009) Effects of glycosylation on the stability of protein pharmaceuticals. J Pharm Sci 98(4):1223–1245. doi:10.1002/jps.21504
2. Kawasaki N, Itoh S, Hashii N, Takakura D, Qin Y, Huang X, Yamaguchi T (2009) The significance of glycosylation analysis in development of biopharmaceuticals. Biol Pharm Bull 32(5):796–800
3. Li H, d'Anjou M (2009) Pharmacological significance of glycosylation in therapeutic proteins. Curr Opin Biotechnol 20(6):678–684. doi:10.1016/j.copbio.2009.10.009
4. Yamada K, Kakehi K (2011) Recent advances in the analysis of carbohydrates for biomedical use. J Pharm Biomed Anal 55(4):702–727
5. Mechref Y (2011) Analysis of glycans derived from glycoconjugates by capillary electrophoresis–mass spectrometry. Electrophoresis 32(24):3467–3481. doi:10.1002/elps.201100342
6. Pioch M, Bunz S-C, Neusüß C (2012) Capillary electrophoresis/mass spectrometry relevant to pharmaceutical and biotechnological applications. Electrophoresis 33(11):1517–1530. doi:10.1002/elps.201200030
7. Schwarzer J, Rapp E, Reichl U (2008) N-glycan analysis by CGE-LIF: profiling influenza A virus hemagglutinin N-glycosylation during vaccine production. Electrophoresis 29(20):4203–4214
8. Khandurina J, Blum DL, Stege JT, Guttman A (2004) Automated carbohydrate profiling by capillary electrophoresis: a bioindustrial approach. Electrophoresis 25(14):2326–2331
9. Kamoda S, Ishikawa R, Kakehi K (2006) Capillary electrophoresis with laser-induced fluorescence detection for detailed studies on N-

linked oligosaccharide profile of therapeutic recombinant monoclonal antibodies. J Chromatography A 1133(1–2):332–339
10. Ruhaak L, Zauner G, Huhn C, Bruggink C, Deelder A, Wuhrer M (2010) Glycan labeling strategies and their use in identification and quantification. Anal Bioanal Chem 397(8):3457–3481. doi:10.1007/s00216-010-3532-z
11. Gennaro LA, Salas-Solano O (2008) On-line CE-LIF-MS technology for the direct characterization of N-linked glycans from therapeutic antibodies. Anal Chem 80(10):3838–3845
12. Szabo Z, Guttman A, Rejtar T, Karger BL (2010) Improved sample preparation method for glycan analysis of glycoproteins by CE-LIF and CE-MS. Electrophoresis 31(8):1389–1395
13. Mechref Y, Novotny MV (2009) Glycomic analysis by capillary electrophoresis–mass spectrometry. Mass Spectrom Rev 28(2):207–222
14. Guttman A, Chen F-TA, Evangelista RA (1996) Separation of 1-aminopyrene-3,6,8-trisulfonate-labeled asparagine-linked fetuin glycans by capillary gel electrophoresis. Electrophoresis 17(2):412–417
15. Riekkola M-L, Jönsson JÅ, Smith RM (2004) Terminology for analytical capillary electromigration techniques. Pure Appl Chem 76(2):443–451
16. Novotny MV, Soini HA, Mechref Y (2008) Biochemical individuality reflected in chromatographic, electrophoretic and mass-spectrometric profiles. J Chromatogr B 866(1–2):26–47. doi:10.1016/j.jchromb.2007.10.007
17. Campa C, Coslovi A, Flamigni A, Rossi M (2006) Overview on advances in capillary electrophoresis–mass spectrometry of carbohydrates: a tabulated review. Electrophoresis 27(11):2027–2050
18. Amon S, Zamfir AD, Rizzi A (2008) Glycosylation analysis of glycoproteins and proteoglycans using capillary electrophoresis–mass spectrometry strategies. Electrophoresis 29(12):2485–2507
19. Sandra K, Van Beeumen J, Stals I, Sandra P, Claeyssens M, Devreese B (2004) Characterization of cellobiohydrolase I N-glycans and differentiation of their phosphorylated isomers by capillary electrophoresis-Q-Trap mass spectrometry. Anal Chem 76(19):5878–5886
20. Jayo RG, Li J, Chen DDY (2012) Capillary electrophoresis mass spectrometry for the characterization of *O*-acetylated N-glycans from fish serum. Anal Chem 84(20):8756–8762. doi:10.1021/ac301889k
21. Albrecht S, Schols HA, Heuvel EGHM, Voragen AGJ, Gruppen H (2010) CE-LIF-MSn profiling of oligosaccharides in human milk and feces of breast-fed babies. Electrophoresis 31(7):1264–1273
22. Liu Y, Salas-Solano O, Gennaro LA (2009) Investigation of sample preparation artifacts formed during the enzymatic release of N-linked glycans prior to analysis by capillary electrophoresis. Anal Chem 81(16):6823–6829. doi:10.1021/ac9010588
23. Maxwell EJ, Ratnayake C, Jayo R, Zhong X, Chen DDY (2011) A promising capillary electrophoresis–electrospray ionization–mass spectrometry method for carbohydrate analysis. Electrophoresis 32(16):2161–2166. doi:10.1002/elps.201100027
24. Balaguer E, Neusuess C (2006) Glycoprotein characterization combining intact protein and glycan analysis by capillary electrophoresis–electrospray ionization–mass spectrometry. Anal Chem 78(15):5384–5393
25. Ongay S, Neusüβ C (2010) Isoform differentiation of intact AGP from human serum by capillary electrophoresis–mass spectrometry. Anal Bioanal Chem 398(2):845–855. doi:10.1007/s00216-010-3948-5
26. Ruhaak LR, Huhn C, Waterreus W-J, de Boer AR, Neusuess C, Hokke CH, Deelder AM, Wuhrer M (2008) Hydrophilic interaction chromatography-based high-throughput sample preparation method for N-glycan analysis from total human plasma glycoproteins. Anal Chem 80(15):6119–6126
27. Briggs JB, Keck RG, Ma S, Lau W, Jones AJS (2009) An analytical system for the characterization of highly heterogeneous mixtures of N-linked oligosaccharides. Anal Biochem 389(1):40–51. doi:10.1016/j.ab.2009.03.006
28. Chiesa C, O'Neill RA (1994) Capillary zone electrophoresis of oligosaccharides derivatized with various aminonaphthalene sulfonic acids. Electrophoresis 15(1):1132–1140
29. Ruhaak LR, Hennig R, Huhn C, Borowiak M, Dolhain RJEM, Deelder AM, Rapp E, Wuhrer M (2010) Optimized workflow for preparation of APTS-labeled N-glycans allowing high-throughput analysis of human plasma glycomes using 48-channel multiplexed CGE-LIF. J Proteome Res 9(12):6655–6664. doi:10.1021/pr100802f
30. Walker SH, Papas BN, Comins DL, Muddiman DC (2010) Interplay of permanent charge and hydrophobicity in the electrospray ionization of glycans. Anal Chem 82(15):6636–6642. doi:10.1021/ac101227a

Manuscript 3

Capillary electrophoresis/mass spectrometry of APTS-labeled glycans for the identification of unknown glycan species in capillary electrophoresis/laser induced fluorescence systems

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Analytical Chemistry, 2013, DOI: 10.1021/ac401930j

The manuscript presents a CE-MS method for APTS-labeled glycans. The method enables the signal assignment of CE-MS to CSE-LIF thus, can be used for eased identification of unknown glycans in CSE-LIF. The method was optimized in terms of separation efficiency and robustness. Additionally, a comparison to standard CSE-LIF approaches and the alkaline CE-MS method, proposed in manuscript 2, is presented.

Candidate`s work:	Development of acidic CE-TOF MS system for the analysis of APTS-labeled glycans, sample preparation, method validation, comparison with alternative CE-MS systems, application to pharmaceutical products, data processing and interpretation, manuscript preparation

analytical chemistry

Article

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Capillary Electrophoresis/Mass Spectrometry of APTS-Labeled Glycans for the Identification of Unknown Glycan Species in Capillary Electrophoresis/Laser-Induced Fluorescence Systems

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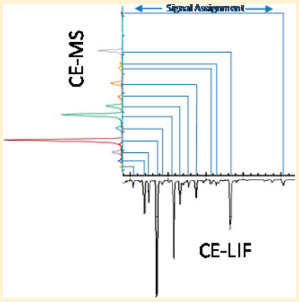
ABSTRACT: The examination of protein glycosylation is of high importance, especially in the (bio)pharmaceutical sector. The analysis of protein glycosylation is conducted routinely in high performance by capillary electrophoresis with laser-induced fluorescence (CE/LIF) using 8-aminopyrene-1,3,6-trisulfonic acid (APTS)-labeled glycans. In this work we present an optimized capillary electrophoresis/time-of-flight mass spectrometry (CE/TOF-MS) methodology for these labeled glycans, which combines the high separation performance of CE with the high resolution, accuracy, and speed of TOF-MS for eased glycan identification. The system based on an acidic background electrolyte (BGE) provides a migration direction analogue to routine CE/LIF systems. Different BGE compositions, capillary dimensions, coatings, and instrumental parameters were tested to optimize the system with respect to separation efficiency and robustness. Subsequently, the CE/MS method optimized for acidic conditions was compared to an alkaline CE/MS method. Further, the mobilities of six APTS-labeled complex-type N-glycans were compared for both CE/MS methods and two standard CE/LIF approaches. For the acidic and alkaline BGE systems, the mobilities of sialylated glycans were shifted relative to nonsialylated glycans in comparison to common CE/LIF systems. However, in this study a straightforward unequivocal peak assignment was achieved for all unknown glycans in a medium complex glycan mixture from a fusion protein.



Glycosylation is one of the most abundant and complex posttranslational modifications. Many protein-based biopharmaceuticals (e.g., monoclonal antibodies, fusion proteins, or cytokines) are glycosylated. It is proven that glycosylation influences folding, stability, and activity of glycoproteins and thus the efficacy of such biopharmaceutical products.[1] During manufacturing, the glycosylation pattern of recombinant glycoproteins (such as monoclonal antibodies (mAb)) may be affected along up- and downstream processing steps,[2,3] as the non-template-driven posttranslational process of glycosylation depends not only on host-cell glycosylation machinery[3] but also on cultivation conditions and the extracellular environment (e.g., cultivation media, temperature, aeration, pH, etc.).

Thus, the analysis and routine control during production of glycoprotein-based drugs is mandatory. A variety of analysis techniques exist based on intact glycoproteins, glycopeptides, or glycans.[4] The entire complexity of glycosylation (including isomers) often requires the analysis of the glycans after cleavage of the amino acid sequence. Therefore, different methods including reversed and normal phase HPLC,[5] hydrophilic interaction chromatography (HILIC),[6] capillary electrophoresis (CE),[7] or high pH anion exchange chromatography (HPAEC)[8] with electrochemical, optical, or mass spectrometric detection have been reported. Among them, CE is a highly efficient and fast tool to separate structural glycan isomers in complex glycan samples. Especially capillary sieving electrophoresis-laser-induced fluorescence detection (CSE/LIF, often referred to as CGE-LIF (capillary gel electrophoresis-laser-induced fluorescence detection), a term used according to IUPAC nomenclature[9])[10−13] is widespread for routine glycan analysis. Different systems based on pseudogel electrolytes have been reported and applied successfully in high throughput analysis in the pharmaceutical and food industry[14] as well as in clinical screening.[12,15] The detection with fluorescence requires sample pretreatment with fluorescence markers, 8-aminopyrene-1,3,6-trisulfonic acid (APTS) being one of the most commonly applied labels for glycans.[16,17] Standard CSE/LIF approaches for APTS-labeled glycan analysis were performed, applying a negative potential to the capillary inlet. Thereof, two powerful systems are based on commercial pseudogel systems.[10,12,13] The methods provide high resolving power, which enables the required separation of structural glycan isomers. However, glycan identification and characterization using optical detection is initially not possible, rather only via matching migration times (normalized to internal standards) with database entries related to the respective glycostructures.[10,12,18]

A powerful alternative here is CE coupled to mass spectrometry (MS), as it combines the high separation power of CE with the selectivity and identification capability of MS.

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Coupling CSE to MS is not feasible, as due to their nonvolatile matter, common CSE electrolytes are not compatible with ESI-MS analysis. Thus, only few CE/MS methods for glycan analysis[19] and for APTS-labeled glycans[14,17,20] have been published so far. A reliable comparison, in terms of the separation efficiencies of the proposed systems, is rather difficult, as it depends on the applied type of glycans. To our knowledge, only Gennaro et al. presented a high resolving separation by CE/MS for APTS-labeled N-glycans of a therapeutic antibody.[17] Recently we developed an alternative background electrolyte (BGE) system under alkaline conditions with anodic detection by MS and high resolving power.[21] To ease the assignment of CE/MS to CSE/LIF signals, analogue migration order is required. Thus, in this work a CE/MS method based on an acidic background electrolyte using a cathodic detection analogue to CSE/LIF is presented. The method was validated for the analysis of glycans with respect to linearity, LODs, and precision. The results were compared with those obtained by two standard CSE/LIF methods and by the alkaline CE/MS method.

MATERIALS AND METHODS

Materials. All solutions were freshly prepared using ultrapure water (18 MΩ cm at 25 °C, SG Ultra Clear UV from Siemens Water Technologies, Alpharetta, GA). Acetonitrile, methanol, and 2-propanol (each LC-MS grade), acetic acid (p.a.), and sodium hydroxide (p.a.) were purchased from Carl Roth GmbH and Co. KG (Karlsruhe, Germany). Citric acid monohydrate, ε-aminocaproic acid (EACA), sodium cyanoborohydride ($NaCNBH_3$) in 1.0 M tetrahydrofuran, and triethylamine, ≥99.5% (TEA) were from Sigma-Aldrich, (Steinheim, Germany). ES Tuning mix solution was purchased from Agilent Technologies (Waldbronn, Germany). The glycan separation gel buffer (GEL1) was purchased from Beckman Coulter (Krefeld, Germany) and the POP-7 polymer (GEL2) from Applied Biosystems (Life Technologies Ltd., Darmstadt, Germany). APTS-labeled glycan standards (G0F, G1F, G1F′, G2F, A1F, and A2F; see structures in Figure 1) were supplied from Ludger (Abingdon, UK), and APTS-labeled glycans of a recombinant fusion protein were from Merck Serono (Rome, Italy).

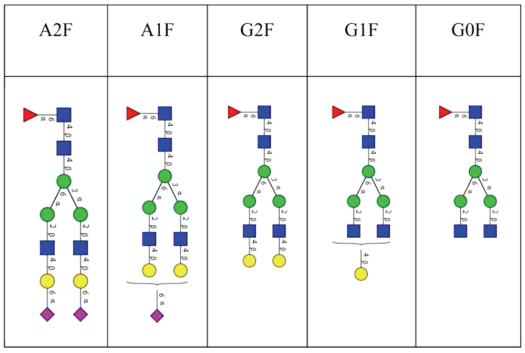

Figure 1. Structures of all glycan standards used in this work. The glycan structures are displayed according to CFG nomenclature. Blue square: *N*-acetylgalactosamine, green circle: mannose, yellow circle: galactose, red triangle: fucose, violet diamond: *N*-acetylneuraminic acid.

Capillaries and Coating. Different neutral coated capillaries with 50 μm inner diameter (i.d.) and 365 μm outer diameter (o.d.) were investigated. Four commercial capillaries with static coating: C-HO capillary 65 cm length (polyvinyl alcohol (PVA) based, Beckman Coulter); eCAP neutral capillary of 67 cm length (polyacrylamide (PAA) based, Beckman Coulter); PVA capillary, originally length 125 cm (used length 80−90 cm) (Agilent Technologies); Guarant of 67 cm length (Alcor Bioseparations, Palo Alto, CA). Further, bare fused silica capillaries from Polymicro Technologies (Phoenix, AZ/distributed by Optronis GmbH, Kehl, Germany) were treated with the neutral dynamic UltraTrol "LN" coating ("low normal" PAA-based precoat) from Target Discovery (Palo Alto, CA). The coating procedure was carried out by flushing these capillaries for 15 min with precoat LN solution and 10 min with water. Other fused silica capillaries of 90 cm length (if not other specified) were PVA coated according to Belder et al.[22]

CE. CE/MS and CE/LIF experiments were performed on a G7100 instrument (Agilent Technologies, Waldbronn, Germany) or a PA800 instrument (Beckman Coulter, Brea, CA). For both setups, separation was performed in PVA capillaries of 90 cm length (o.d. 365 μm, i.d. 50 μm from Polymicro) at −30 kV (inlet) using a background electrolyte of 40 mM EACA, 131 mM acetic acid at a pH of 4. The capillary was flushed with water before the first use or after storage and flushed with air for longer storage. The sample was injected hydrodynamically applying 50 mbar for 12 s. CSE/LIF experiments were performed applying the here-proposed acidic BGE system and the Beckman glycan separation buffer (GEL1) in PVA-coated capillaries or the POP-7 polymer (GEL2) in bare fused silica capillaries, each of 70 cm length (effective length 60.5 cm). Electrokinetic injection at −7 kV for 10 s was used for the highly viscous GEL2.

MS. MS experiments were performed on a QTOF instrument from Bruker Daltronik (microTOFQ, Bremen, Germany). Electrospray ionization was used in negative ion mode applying 4.0 kV on the MS inlet (sprayer on ground). A nebulizer gas pressure of 0.2 mbar was applied to assist the spraying process. For CE/MS coupling, a coaxial sheath−liquid interface was used (CE/MS alignment interface, Agilent Technologies). Sheath−liquid (SL) was optimized and consists of 0.025% TEA in 2-propanol/water 1:1 (v:v). A SL flow rate of 4 μL/min was used. Dry gas (nitrogen, >99.5%) was delivered at 4.0 L/min and 170 °C. MS spectra were acquired in a mass range of m/z 300−2500 at a repetition rate of 1 Hz (summation of 5000 single spectra). The ion transfer was optimized for the required mass range. An external calibration was performed by infusing tuning mix solution at 4 μL/min every day prior to measurements. The identification of glycans was based on the accurate mass of doubly and triply charged glycans with an acceptable mass deviation (<5 ppm). Data analysis was performed using DataAnalysis Version 4.0 from Bruker Daltronik (Bremen, Germany). The glycan identification was verified by comparison of the isotopic mass distribution.

LIF. The laser source was an argon ion laser (488 nm) with an optical power of 3 mW (Beckman Coulter, Brea, CA) applied to the PA800 CE-instrument from Beckman. The CE/LIF/MS setup was applied to the Agilent CE-instrument as described by Huhn et al.[23] Briefly, the detection of fluorescence intensity was performed with a photomultiplier system and a detection cell from J&M Analytik AG (Aalen, Germany). A

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homemade capillary holder was used to place the detection cell right on top of the sheath liquid interface. The excitation light was absorbed by an optical filter (GG495) from Beckman Coulter transmitting wavelengths above 512 nm.

RESULTS AND DISCUSSION

CE/MS Method Development. The aim was the development of a CE/MS method comparable with standard CSE/LIF to ease the identification of unknown glycans. Therefore, the same polarity (negative at inlet) was favored to obtain analogue glycan migration order. Different acidic systems, based on formic acid and acetic acid, were examined. APTS-labeled glycan samples were applied to formic acid-based BGE systems as described by Albrecht et al.[14] (3% formic acid) or a previously presented system for sulfonic acids[24] (0.2 M formic acid). Additionally, acetic acid in different concentrations (0.2–0.5 M) was examined as the BGE. No satisfactory separations were obtained (data not shown) especially when compared to the CE/LIF/MS method published by Gennaro et al.,[17] which met all requirements with respect to separation (i.e., high separation efficiency, analogue migration order by anodic detection). However, the performance of that method (based on a BGE of EACA) could not be reproduced in our laboratory, especially with respect to peak shape and separation efficiency. This is probably due to the pressure sensitivity influences (e.g., siphoning, ESI-sprayer status, and nebulizer gas flow rate) as already mentioned by Gennaro et al. Because of these findings, the CE/LIF/MS method previously presented by Gennaro et al.,[17] had to be modified to increase performance for CE/MS analysis of APTS-labeled glycans. So, in a first screening, the CE/MS parameters expected to have major impact on separation efficiency and stability of the method were evaluated in a design of experiments (DOE) and varied around the parameters described by Gennaro et al. (i.e., 40 mM EACA at pH 4.5 and SL of 0.2% ammonia in 2-propanol/water (1:1, v:v)).[17] An initially preformed variation of the EACA concentration (between 20 and 80 mM) could not improve the results (data not shown) and was not taken into account for the DOE. The pH of the BGE (adjusted with acetic acid) and potential addition of ammonia to the BGE were in addition to the remaining ammonia concentration testing as well as pH and solvent composition of the in the BGE and SL (influence on pH), and the SL solvent composition testing (i.e., ratio of 2-propanol to water). Finally, the nebulizer gas flow and the influence of capillary position in the CE-ESI sprayer were examined (pressure variation). Each factor was set at two levels as presented in Table 1. Each parameter combination and the method of Gennaro et al. were repeated three times in randomized order. Altogether 54 experiments were carried out to test the different parameter combinations (data not shown). These experiments were performed on two commercial PVA capillaries, applying an APTS-labeled G1F-standard (two isomeric asialo-, biantennary complex-type N-glycans with one galactose attached to either end of the antennae; see structure in Figure 1).

Table 1. Applied Levels for Evaluating Factor Influences

factor	level 1	level 2
pH of BGE (acetic acid)	4	5
ammonia concentration in BGE (mM)	0	10
SL composition (2-propanol/water)	50/50	20/80
ammonia concentration in SL (%)	0.05	0.35
nebulizer gas pressure (bar)	0.2	0.5
capillary position at sprayer outlet	lined-up	0.2 mm out

In this DOE-driven first screening, minor effects were observed for pH and ammonia content of BGE as well as capillary position at the sprayer outlet. The optimal BGE was found to be 40 mM EACA at a pH of 4 (131 mM acetic acid) without the addition of ammonia. The nebulizer gas pressure of 0.2 bar showed significantly higher separation efficiency. A 50/50 SL composition of 2-propanol/water was more stable than 20/80. The ammonia concentration in the SL had a major impact. On the basis of the DOE results, the main contributing parameters (SL composition and pressure effects) were studied in detail as discussed in the following sections. Additionally, the temperature influence turned out to be of importance and was tested as well for different capillary coatings.

Optimization of SL Composition. For the BGE system based on 40 mM EACA at a pH of 4, different SL compositions were tested (data not shown). Different organic solvents such as methanol or acetonitrile decreased the ionization efficiency for APTS-labeled glycans compared to 2-propanol. Thus, the SL was composed of 2-propanol at concentrations between 20% and 50% with different acidic (formic and acetic acid) and basic additives (ammonia, aniline, and triethylamine (TEA)). These experiments demonstrated that basic additives are required to achieve good separation efficiency. The positively charged amines from the SL that enter the separation capillary from the outlet seem to enhance the separation efficiency, possibly because moving ion boundaries and/or ion interactions are created.[25] An increase in current was observed as a side effect (i.e., from 12 μA to 22 μA in 10 min with 0.2% ammonia in SL). However, using TEA or aniline with a stoke radius larger than that of ammonia, and thus lower electrophoretic mobilities, a reduced current rise was observed (data not shown). Finally, TEA enabled the best separation efficiency in combination with a reduced current increase (only about 1.5 μA in 10 min). Thus, the optimal composition of SL was 0.025% TEA in 50% 2-propanol/water (v:v).

Influence of Pressure. The separation in this system showed sensitivity to pressure variation on the CE, because in CE/MS systems with no EOF the only transport mechanism is the electromigration of ions, and pressure control at the outlet vial is missing. Therefore, the nebulizer gas flow, pressure, or vacuum applied during the run on the inlet vial as well as possible siphoning effects can affect analysis performance. The lowest value for nebulizer gas flow (0.2 bar) was found to be best, maintaining a good and stable electrospray without affecting the separation. The influence of applying pressure or vacuum to the capillary inlet is depicted in Figure 2; the experiments were performed by CE/LIF on a PVA capillary of 90 cm length (L_{eff} = 80 cm) using the described acidic BGE. The outlet vial was filled with SL. Small pressure differences of 10 mbar have a strong effect on glycan separation. Here, in CE/LIF, the optimal value of additional pressure was found to be −10 mbar (see Figure 2). Furthermore, it was found that the sprayer status (i.e., corrosion or other damages on the sprayer tip) and spray needle position may affect the pressure balance (suction effect). Therefore, it is recommended to adjust the inlet pressure to achieve the best separation efficiency.

Influence of Temperature. The temperature control of CE-capillaries is difficult in CE/MS experiments because the capillary is guided out of the CE-instrument and the capillary end (~10 cm) is located inside the ESI sprayer. Thus, for low

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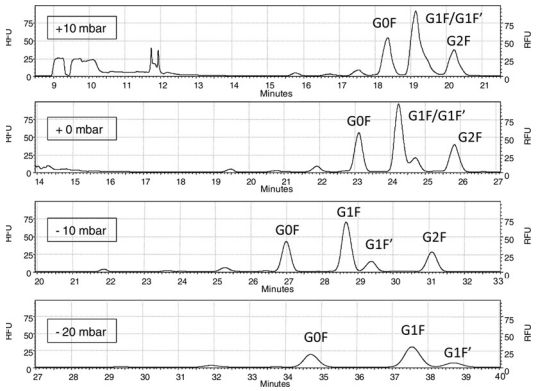

Figure 2. CE/LIF electropherograms of an APTS-labeled glycan sample of a recombinant fusion protein at different inlet pressure values. The proposed acidic method was applied in a PVA capillary of 90 cm length and SL in the outlet vial.

CE-current values in MS applications (<50 μA), cooling is not performed. However, here an influence of ambient temperature was observed; thus, the effects were studied, varying temperature between 15 °C and 27.5 °C in a temperature-controlled CE/LIF setup. The temperature values were set in random order in a PVA-capillary of 1 m length. The reproducibility was proven by running the 25 °C experiment twice. The migration times of G1F shifted about 2 min per 2.5 °C as shown in Figure 3. Thus, to avoid changes in migration times, the ambient temperature should be controlled.

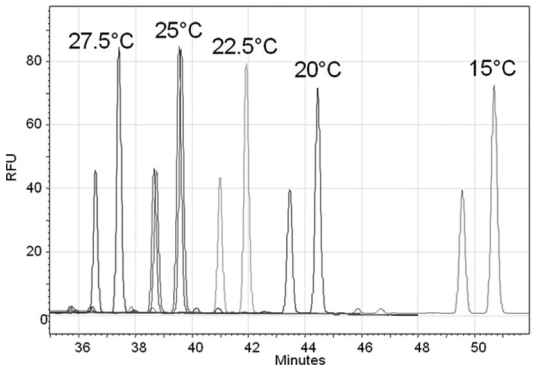

Figure 3. Overlaid CE/LIF electropherograms of an APTS-labeled G1F standard measured at different temperatures (the 25 °C experiment twice). All experiments were performed in randomized order using the proposed acidic method in a CE/LIF setup.

Neutral Coated Capillaries. Electroosmotic backflow at pH 4.0 needs to be omitted to prevent SL from entering the CE-capillary. Therefore, different dynamic and permanent neutral coatings, such as PVA, PAA, and Guarant as well as a neutral dynamic coating based on soluble PAA ("LN"), were tested. The separations on all these neutral coated capillaries were similar when using identical capillary lengths. For the dynamic "LN", a recoating every 4 runs was required while the capillary had to be removed from MS. Permanent coating simplifies the routine analysis, but only a few suppliers provide permanent neutral coated capillaries longer than 80 cm, which is essential for the separation of structural glycan isomers using the proposed method. Thus, homemade PVA-capillaries were compared to commercial PVA-capillaries. For homemade PVA-capillaries, no changes in migration time and efficiency were observed during 36 runs. A migration time precision of 4% (RSD) between different PVA-coated capillaries (two commercial and four homemade capillaries) was determined. Hence, the reliability of the coating procedure and the comparability of homemade with commercial capillaries were corroborated.

Influence of the ESI-Interface on Separation Efficiency. The general influence of the MS-interface was examined by an in-line CE/LIF/MS setup. In Figure 4 the electropherograms with LIF and MS detection of separated APTS-labeled glycans derived from a recombinant fusion protein are presented. The detection of G0F, G1F, and G2F demonstrated similar peak widths and separation efficiency for both detectors. Thus, in our case separation was not affected by the electrospray during the spray event. However, separation efficiency is lower in CE/LIF/MS (resolution: $R_{G1F/G1F'}$ = 1.1) when compared to CE/LIF-only with SL in outlet vial ($R_{G1F/G1F'}$ = 2.5), possibly caused by the remaining pressure effects of, for example, the nebulizer gas flow.

Method Validation. Under the optimal conditions in a PVA capillary, i.e., BGE of 40 mM EACA at pH 4.0 adjusted with acetic acid (131 mM acetic acid) and SL consisting of 50% 2-propanol and 0.025% TEA, the CE/MS method was validated using an APTS-labeled A1F glycan standard. The concentration of labeled A1F was calculated, taking into account a labeling efficiency of 42% as determined by using the alkaline BGE system.[21] Linear regression was performed in a range of 5−20 μM with a coefficient (r) of 0.9982. An LOD of 0.6 μM was determined for S/N of 3. Further validation experiments are presented in the next paragraph in comparison with an alternative alkaline system.

Comparison of Alkaline and Acidic Methods. Recently, an alkaline BGE system for APTS-labeled and charged (sialylated) glycans, with good results in terms of structural isomer separation, was presented by our group.[21] A BGE of 0.7 M ammonia, 0.1 M ε-aminocaproic acid in methanol/water (7:3, v:v), a fused silica capillary of 90 cm length with cathodic detection (+30 kV inlet), and sheath liquid of 50% 2-propanol/water was applied.

The repeatability of the acidic and alkaline systems in capillaries of 90 cm length was compared, applying APTS-labeled glycans derived from a recombinant fusion protein. The RSD of migration time and relative peak intensity for G1F as well as the average resolution of two isomeric G1F peaks based on the full-width-half-maximum (fwhm) were calculated by 11 replicates in the acidic and 12 replicates in the alkaline system, respectively (data not shown). The migration time intraday precisions were 2.6% (10.8% interday) and 3.2% (4.7% interday) (RSDs) in the acidic and the alkaline BGE systems, respectively. The relative peak intensity of G1F peaks showed a intraday RSD of 6.1% (acidic, 7.1% interday) and 5.0% (alkaline, interday 6.9%), respectively. The average resolutions (R) of G1F isoforms were comparable in both systems (R = 1.2 (acidic) and R = 1.1 (alkaline)). On the other hand, higher signal intensity was found for the alkaline CE/MS system (increased peak height by a factor of 3−8). It seems that the high percentage of methanol in the alkaline BGE supports the ionization process. Additionally, the ionization of APTS-labeled glycans was decreased in the acidic system due to the

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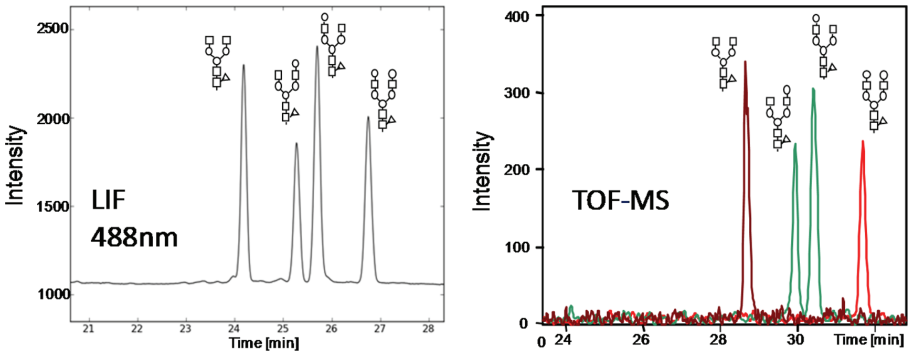

Figure 4. LIF trace of G0F, G1F, and G2F standards (left); EIE of G0F, G1F, and G2F (right) by TOF-MS. □: *N*-Acetylhexosamine, ○: hexose, Δ: fucose, ◇: sialic acid. The CE/LIF/MS separation was performed on an LN capillary of 98 cm length with 40 mM EACA at pH 4 and sheath liquid of 20% 2-propanol + 0.025% TEA.

requirement of basic additives in the sheath liquid (i.e., TEA). Peak height was decreased by a factor of 3 when the TEA-containing sheath liquid was applied to the alkaline CE/MS system (data not shown). Nevertheless, the method was successfully applied to characterize minor glycan species in the recombinant fusion protein. Thirteen out of 14 peaks detected by CSE/LIF could be identified by CE/MS, reaching down to the 0.7% level (compared to the intensity of the main glycan). This shows good applicability of the presented approach to pharmaceutically relevant glycoproteins.

Comparison of Glycan Mobilities in CE/MS and CSE/LIF Methods. As mobilities might change in CE, because of different BGEs (e.g., due to BGE pH and pK_as of the analytes, solvation of the analytes, BGE viscosity), a comparison between the developed acidic and alkaline CE/MS methods and two CSE/LIF methods (using GEL1 and GEL2) was performed with respect to glycan mobilities. The four methods were applied to capillaries of 70 cm length (effective length 60.5 cm) using LIF detection. The CE/MS methods were simulated via CSE/LIF, applying SL in the outlet vial. The methods were compared using APTS-labeled biantennary, core-fucosylated complex-type glycans: four nonsialylated (G0F, G1F, G1F′, G2F; see structures in Figure 1) and two sialylated (A1F, A2F; see structures in Figure 1). The analyte's mobilities in the acidic BGE, GEL1, and GEL2 were determined assuming a negligible EOF (neutral coated capillaries). For the alkaline system, the EOF mobility was determined to be 0.015 $mm^2/(V\ s)$ using rhodamine B as a neutral EOF marker (data not shown).

Figure 5 displays the mobilities in the acidic BGE, GEL2 and alkaline BGE systems (*y*-coordinate) plotted against the mobilities in GEL1 (*x*-coordinate). Trend lines of the nonsialylated glycans demonstrate its linear relation. The general mobility differences between the systems were caused by different BGE properties (Table 2). The lower glycan mobilities in the alkaline BGE and GEL2 in relation to GEL1 are expected to be due to reduced analyte dissociation in methanol and high viscosity, respectively. The higher mobilities in the acidic BGE system are probably due to the lower viscosity, different analyte solvation, and/or ion interactions.

The mobilities of all tested glycans in GEL2 are proportional to the mobilities determined in GEL1. The same is valid only for the nonsialylated glycans when comparing the alkaline and the acidic BGE to the GEL1, respectively. In the alkaline and to a less extent in the acidic system, the mobilities of sialylated glycans differ from the linear behavior of the nonsialylated glycans. Thus, peak shifts and changes in migration order of sialylated and nonsialylated glycans are possible when CSE/LIF and CE/MS methods are compared. However, if most glycan species in a sample are known or the sample is not too complex, unknown glycan peaks are expected to be assignable. On the basis of the glycan analysis of a recombinant fusion protein, the assignment of peaks identified by the alkaline CE/MS method to peaks detected in CSE/LIF (GEL2) is demonstrated (Figure 6). Linear regression is displayed for

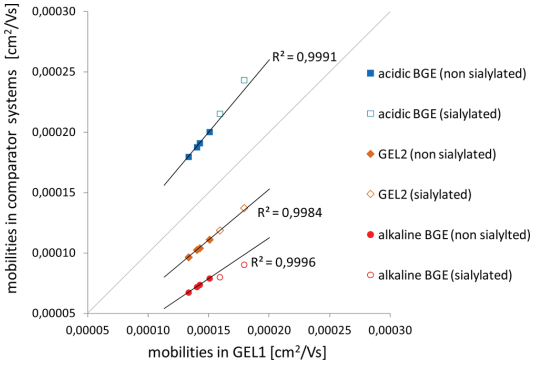

Figure 5. Glycan mobilities of different systems (acidic and alkaline BGE, GEL2) are plotted against the glycan mobilities in GEL1. ■, ◆, ▲: nonsialylated glycans. □, ◇, Δ: sialylated glycans.

Table 2. BGE Properties of Common CSE/LIF Systems and the Presented Acidic and Alkaline CE/MS Systems

	POP-7	Beckman separation buffer	acidic BGE	alkaline BGE
viscosity (mPa·s)	187	3.431	1.907	1.308
pH	10	4.5	4	10 (apparent)
coating	dynamic neutral coated	PVA	PVA	fused silica
polarity	reverse (−30 kV)	reverse (−30 kV)	reverse (−30 kV)	normal (+30 kV)

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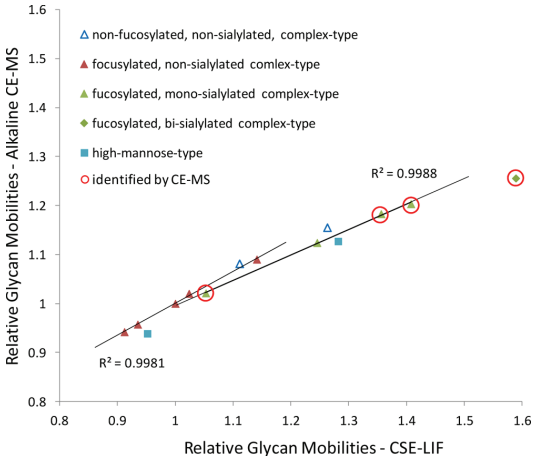

Figure 6. Relative glycan mobilities of the CSE/LIF analysis (GEL2) plotted against the glycan mobilities in the alkaline CE/MS system. The mobilities are normalized to a fucosylated, nongalacatosylated, biantennary complex-type glycan (G0F). The fucosylated, non-sialylated and the fucosylated, monosialylated glycans were regressed, respectively.

the fucosylated, nonsialylated and for the fucosylated, monosialylated glycans (four data points each), respectively. Even though the sample contains a variety of different glycan structures (complex type−high mannose type, sialylated−nonsialylated, fucosylated−nonfucosylated), all peaks identified by CE/MS can be clearly assigned to peaks in the CSE/LIF approach.

CONCLUSIONS

The developed CE/MS method with acidic BGE provides high separation efficiency for APTS-labeled glycans and is applicable for identification of unknown glycans in CSE/LIF. Several method parameters were examined to optimize the method's resolving power and robustness. For good separation efficiency, a basic additive in SL is required. Capillary length of 90 cm was best suited for structural isomer separation, independent of the kind of neutral coating. The proposed method was tested on CE/MS and CE/LIF. A CE/LIF/MS approach showed that resolution is not affected by the electrospray process. Pressure variations (e.g., nebulizer gas flow, capillary position, and sprayer needle status) constituted the main influence on separation efficiency and peak shape. However, flow and pressure effects can be counterbalanced.

In comparison with our previously published alkaline BGE system for APTS-labeled and charged native glycans, the achieved ionization efficiency is 3−8-times lower. However, for the alkaline BGE system, air intake during vial change was observed using the Beckman Coulter instrument, possibly due to the low surface tension of methanol (70%). For both CE/MS methods, the mobilities of sialylated glycans were shifted relative to nonsialylated glycans in comparison to common CSE/LIF systems, as demonstrated here for biantennary, core-fucosylated glycans. Thus, the transfer of structural peak assignment from CE/MS to CSE/LIF may be challenging in very complex samples. However, in this study unequivocal peak assignment was achieved. Known glycan benchmarks or glycan mobility databases may enable the assignment of peaks identified by CE/MS to CSE/LIF peaks even for complex samples.

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Notes

The authors declare no competing financial interest.

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NOMENCLATURE

APTS	8-aminopyrene-1,3,6-trisulfonic acid
CFG	Consortium for Functional Glycomics
CSE	capillary sieving electrophoresis
DOE	design of experiments
EACA	ε-aminocaproic acid
HPAEC	high pH anion exchange chromatography
PAA	polyacrylamide
PVA	polyvinyl alcohol
R	resolution
SL	sheath liquid
TEA	triethylamine

REFERENCES

(1) Kawasaki, N.; Itoh, S.; Hashii, N.; Takakura, D.; Qin, Y.; Huang, X.; Yamaguchi, T. *Biol. Pharm. Bull.* **2009**, *32*, 796−800. Li, H.; d'Anjou, M. *Curr. Opin. Biotechnol.* **2009**, *20*, 678−684. Jefferis, R. *Nat. Rev. Drug Discovery* **2009**, *8*, 226−34. Sola, R. J.; Griebenow, K. *J. Pharm. Sci.* **2009**, *98*, 1223−45.

(2) Sheeley, D. M.; Merrill, B. M.; Taylor, L. C. *Anal. Biochem.* **1997**, *247*, 102−10. Jenkins, N.; Parekh, R. B.; James, D. C. *Nat. Biotechnol.* **1996**, *14*, 975−81. Butler, M.; Huzel, N.; Yang, M.; Restelli, V.; Kunkel, J. *Abstracts of Papers*, 225th National Meeting of the American Chemical Society, New Orleans, LA, March 23−27, 2003; American Chemical Society: Washington, DC, 2003; U209. Gawlitzek, M.; Valley, U.; Nimtz, M.; Wagner, R.; Conradt, H. S. *J. Biotechnol.* **1995**, *42*, 117−131.

(3) Jenkins, N.; Curling, E. M. A. *Enzyme Microb. Technol.* **1994**, *16*, 354−364. Goochee, C. F. *Developments in Biological Standardization* **1992**, *76*, 95−104.

(4) Pioch, M.; Bunz, S.-C.; Neusüß, C. *Electrophoresis* **2012**, *33*, 1517−1530. Rakus, J. F.; Mahal, L. K. *Annu. Rev. Anal. Chem.* **2011**, *4*, 367−392.

(5) Huhn, C.; Selman, M. H. J.; Ruhaak, L. R.; Deelder, A. M.; Wuhrer, M. *Proteomics* **2009**, *9*, 882−913. Prater, B. D.; Connelly, H. M.; Qin, Q.; Cockrill, S. L. *Anal. Biochem.* **2009**, *385*, 69−79.

(6) Wuhrer, M.; Boer, A. R. d.; Deelder, A. M. *Mass Spectrom. Rev.* **2009**, *28*, 192−206. Melmer, M.; Stangler, T.; Schiefermeier, M.; Brunner, W.; Toll, H.; Rupprechter, A.; Lindner, W.; Premstaller, A. *Anal. Bioanal. Chem.* **2010**, *398*, 905−914. Ruhaak, L. R.; Huhn, C.; Waterreus, W.-J.; de Boer, A. R.; Neusüss, C.; Hokke, C. H.; Deelder, A. M.; Wuhrer, M. *Anal. Chem.* **2008**, *80*, 6119−6126.

(7) Nakano, M.; Kakehi, K.; Taniguchi, N.; Kondo, A. *Capillary Electrophoresis of Carbohydrates: From Monosaccharides to Complex Polysaccharides*; Humana Press: Totowa, NJ, 2011; pp 205−235. Mechref, Y. *Electrophoresis* **2011**, *32*, 3467−3481. Pattky, M.; Huhn, C.

dx.doi.org/10.1021/ac401930j | *Anal. Chem.* XXXX, XXX, XXX−XXX

Bioanal. Rev. **2010**, *2*, 115−155. Amon, S.; Zamfir, A. D.; Rizzi, A. *Electrophoresis* **2008**, *29*, 2485−2507.
(8) Behan, J. L.; Smith, K. D. *Biomed. Chromatogr.* **2011**, *25*, 39−46. Grey, C.; Edebrink, P.; Krook, M.; Jacobsson, S. P. *J. Chromatogr., B* **2009**, *877*, 1827−1832. Guignard, C.; Jouve, L.; Bogeat-Triboulot, M. B.; Dreyer, E.; Hausman, J.-F.; Hoffmann, L. *J. Chromatogr., A* **2005**, *1085*, 137−142. Bruggink, C.; Maurer, R.; Herrmann, H.; Cavalli, S.; Hoefler, F. *J. Chromatogr., A* **2005**, *1085*, 104−109.
(9) Riekkola, M.-L.; Jönsson, J. Å.; Smith, R. M. *Pure Appl. Chem.* **2004**, *76*, 443−451.
(10) Schwarzer, J.; Rapp, E.; Reichl, U. *Electrophoresis* **2008**, *29*, 4203−4214.
(11) Chen, F.-T. A.; Evangelista, R. A. *Electrophoresis* **1998**, *19*, 2639−2644. Szabo, Z.; Guttman, A.; Bones, J.; Karger, B. L. *Anal. Chem.* **2011**, *83*, 5329−5336. Guttman, A.; Chen, F. T.; Evangelista, R. A. *Electrophoresis* **1996**, *17*, 412−7.
(12) Ruhaak, L. R.; Hennig, R.; Huhn, C.; Borowiak, M.; Dolhain, R. J. E. M.; Deelder, A. M.; Rapp, E.; Wuhrer, M. *J. Proteome Res.* **2010**, *9*, 6655−6664.
(13) Vogel, K.; Kuhn, J.; Kleesiek, K.; Götting, C. *Electrophoresis* **2006**, *27*, 1363−1367. Ma, S.; Nashabeh, W. *Anal. Chem.* **1999**, *71*, 5185−5192.
(14) Albrecht, S.; Schols, H. A.; Heuvel, E. G. H. M. v. d.; Voragen, A. G. J.; Gruppen, H. *Electrophoresis* **2010**, *31*, 1264−1273.
(15) Lee, K. J.; Jung, J.-H.; Lee, J. M.; So, Y.; Kwon, O.; Callewaert, N.; Kang, H. A.; Ko, K.; Oh, D.-B. *Biochem. Biophys. Res. Commun.* **2009**, *380*, 223−229. Callewaert, N.; Geysens, S.; Molemans, F.; Contreras, R. *Glycobiology* **2001**, *11*, 275−281.
(16) Smejkal, P.; Szekrényes, Á.; Ryvolová, M.; Foret, F.; Guttman, A.; Bek, F.; Macka, M. *Electrophoresis* **2010**, *31*, 3783−3786. Ruhaak, L.; Zauner, G.; Huhn, C.; Bruggink, C.; Deelder, A.; Wuhrer, M. *Anal. Bioanal. Chem.* **2010**, *397*, 3457−3481.
(17) Gennaro, L. A.; Salas-Solano, O. *Anal. Chem.* **2008**, *80*, 3838−3845.
(18) Kottler, R.; Mank, M.; Hennig, R.; Müller-Werner, B.; Stahl, B.; Reichl, U.; Rapp, E. *Electrophoresis* **2013**. Rapp, E.; Hennig, R.; Borowiak, M.; Kottler, R.; Reichl, U. *Glycoconjugate J.* **2011**, *28*, 234−235.
(19) Balaguer, E.; Neusüss, C. *Anal. Chem.* **2006**, *78*, 5384−5393. Ongay, S.; Neusü*β*, C. *Anal. Bioanal. Chem.* **2010**, *398*, 845−855. Ruhaak, L. R.; Huhn, C.; Waterreus, W.-J.; de Boer, A. R.; Neusüss, C.; Hokke, C. H.; Deelder, A.; Wuhrer, M. *Anal. Chem.* **2008**, *80*, 6119−6126. Nakano, M.; Higo, D.; Arai, E.; Nakagawa, T.; Kakehi, K.; Taniguchi, N.; Kondo, A. *Glycobiology* **2009**, *19*, 135−143.
(20) Szabo, Z.; Guttman, A.; Rejtar, T.; Karger, B. L. *Electrophoresis* **2010**, *31*, 1389−1395. Liu, Y.; Salas-Solano, O.; Gennaro, L. A. *Anal. Chem.* **2009**, *81*, 6823−6829. Jayo, R. G.; Li, J.; Chen, D. D. Y. *Anal. Chem.* **2012**, *84*, 8756−8762.
(21) Bunz, S.-C.; Cutillo, F.; Neusüß, C. *Anal. Bioanal. Chem.* **2013**.
(22) Belder, D.; Deege, A.; Husmann, H.; Kohler, F.; Ludwig, M. *Electrophoresis* **2001**, *22*, 3813−3818.
(23) Huhn, C.; Ruhaak, L. R.; Mannhardt, J.; Wuhrer, M.; Neusüß, C.; Deelder, A. M.; Meyer, H. *Electrophoresis* **2012**, *33*, 563−566.
(24) Bunz, S.-C.; Weinmann, W.; Neusüß, C. *Electrophoresis* **2010**, *31*, 1274−1281.
(25) Foret, F.; Thompson, T. J.; Vouros, P.; Karger, B. L.; Gebauer, P.; Bocek, P. *Anal. Chem.* **1994**, *66*, 4450−4458.

Manuscript 4

In-line SPE-CE-MS using a fritless bead string design – Application for the analysis of organic sulfonates including APTS-labeled glycans

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The manuscript presents a novel in-line SPE-CE setup. Thereby the sensitivity of the proposed acidic CE-MS method was increased with respect to sample concentration, to enable the detection of samples in the low femto-molar (fM)-range as analyzed in CE-LIF approaches. Applying the proposed in-line SPE-CE-MS system an enrichment factor of 800 was obtained compared to the proposed acidic CE-MS system for the analysis of five APTS-labeled complex-type N-glycans.

Candidate's work:	Initial offline SPE experiments, project assistance, discussion and preparation of manuscript

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Research Article

In-line SPE-CE using a fritless bead string design—Application for the analysis of organic sulfonates including inline SPE-CE-MS for APTS-labeled glycans

Despite many advantages like high separation efficiency CE comprises the main limitation of low concentration sensitivity, when compared to HPLC. In-line SPE is an efficient way to increase concentration sensitivity. Here, a fritless in-line-SPE-CE-MS method was developed in order to analyze anions of strong acids. Mixed-mode (weak anion exchange and RP) particles were used for enrichment and an acidic BGE was applied for separation. Different particle and capillary sizes were tested. A novel bead string design with a 100 μm id column filled with particles of 90 μm followed by a separation capillary with 50 μm id was easy to prepare and showed the best performance with respect to separation efficiency and reproducibility. Three aromatic sulfonic acids were employed in an in-line SPE-CE-UV approach for method development. Method validation was performed with respect to reproducibility, robustness, and linearity. Thereafter the method was transferred to SPE-CE-MS and applied to the analysis of glycans labeled with 8-aminopyrene-1,3,6-trisulfonic acid. Lower limits of detection in the low nM range were achieved injecting about 10 μL of sample. This corresponds to an enrichment factor of more than 800 compared to the corresponding CE-MS method without preconcentration.

Keywords:
CE-MS / Glycans / Inline SPE-CE / Organic sulfonates
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Additional supporting information may be found in the online version of this article at the publisher's web-site

1 Introduction

CE comprises a unique selectivity and other analytical advantages like high separation efficiency. In addition, only a small amount of sample and electrolyte is required, due to the small dimensions of CE. The total volume of the capillaries does not exceed a few micro liters and injection volumes are generally in a range of a few tens of nanoliters (1–2% of the capillary volume). However, this is associated with poor concentration sensitivity, one of the major drawbacks of CE.

There are various techniques to enrich analytes, in order to increase the injection volume and, thus, the concentration sensitivity. Electrophoresis-based enrichment techniques, like various stacking techniques [1], transient ITP [2] or dynamic pH-junction [3] are often easy to implement; however show limited enrichment factors especially if used in CE-MS, where a second buffer vial is missing [4]. Chromatography-based enrichment techniques, that is, in-line SPE enable high enrichment factors and may additionally be used for sample cleanup. Three general approaches to construct an in-line SPE-CE system can be distinguished: (i) open tubular columns (wall coated with SPE sorbent) [5–7] are simple to construct and allow a fast loading, due to the marginal increase in back pressure. However, the surface and, thus, the capacities of such devices are small. (ii) Monolithic in-line SPE-CE capillaries contain a small section of functionalized monolith [8–11] or membrane [12–17]. Advantages of monolithic columns are the high surface area and the absence of frits. However, a quantitative elution requires a large elution volume, in comparison to packed beds [8]. In addition, there are often problems with reproducibility, associated with the process of reproducibly loading and elution [9]. (iii) Packed

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Abbreviations: ad, average diameter; **APTS**, 8-aminopyrene-1,3,6-trisulfonic acid; **BSA**, benzenesulfonic acid; **FAc**, formic acid; **NSA**, naphthalenesulfonic acid; **TSA**, *p*-toluenesulfonic acid

Colour Online: See the article online to view Figs. 1, 3, and 5 in colour.

beds are most commonly used in in-line SPE-CE and have been used in many analytical applications [18–28]. There are different approaches for the construction of a packed bed capillary. Often frits are used to keep the SPE material in place [21, 29–35]. However, the construction of frits is an additional elaborate step and frits may lead to high backpressure or an irreproducible EOF. In addition, frits are vulnerable for bubble formation, disrupting the separation. Therefore, fritless packed bed capillaries have been constructed in recent years [18–20, 22–27, 36]. In general, the capillary consists of three individual pieces. Between an inlet (5–10 cm, e.g. 50 μm id) and a separation capillary (50–100 cm, e.g. 50 μm id), a small piece of capillary (typically 2–4 mm, with a larger id, e.g. 150 μm id) contains the particles with diameters larger than the id of the separation capillary. The joint between the three individual capillary pieces can be accomplished by one [20, 22–24, 26, 28] or two PTFE tubings [18, 19, 25]. Most of these studies used an average SPE particle size of 60 μm by sieving the particles.

Mostly cations like peptides [37–39] or small molecules [25, 28, 40] have been analyzed using in-line SPE-CE. Anions are rarely analyzed [28]. RP-based material is most widely being used for these applications [41–45]. Many other materials for SPE have been developed in the last years. Mixed-mode material is especially useful for the enrichment of polar and ionic analytes and, thus, has been applied in off-line SPE-CE [46–48] and in-line SPE-CE [18, 23, 26]. Bunz et al. [49] used a mixed-mode SPE material (weak anion exchange + reverse phase properties) for off-line-SPE concentration of ethylsulfate and related analytes from urine prior to CE-MS analysis. However, to our knowledge, mixed-mode sorbents have not been applied to analyze anions by in-line SPE-CE.

CE-MS has become a broadly applied technique for the analysis of all kinds of analytes. Recently, a method was developed in our group analyzing selectively the anions of strong acids under acidic conditions [49, 50]. Similarly, glycans as derived from proteins can be analyzed after derivatization with 8-aminopyrene-1,3,6-trisulfonic acid (APTS) [49, 51] by CE-MS in an acidic BGE. These approaches allow the identification of glycans from CE-LIF measurements as routinely applied in the biopharmaceutical context [52, 53]. However, concentration sensitivity for these APTS-labeled glycans is about three orders of magnitude lower for CE-MS compared to CE-LIF.

In this work, we present an in-line SPE-CE-MS method for the analysis of anions of strong acids like APTS-labeled glycans. Extensive experiments for the constructions, the geometries (particle, bed, and column dimensions) and the elution method have been performed in order to find the most easy and reproducible set-up.

2 Materials and methods

2.1 Reagents and solution

2-Propanol, formic acid (FAc), methanol (all LC-MS grade), and aqueous ammonia (30%—p.a.) were purchased from Carl Roth (Karlsruhe, Germany). Acetic acid (LC-MS grade) was obtained from Fisher Scientific (Schwerte, Germany). Triethylamine (≥ 99.5%), epsilon-aminocaproic acid (≥ 99%), and naphtalene-2-sulfonic acid (technical, > 70%) were purchased from Sigma-Aldrich (Steinheim, Germany), benzenesulfonic acid (BSA) and *p*-toluenesulfonic acid (TSA) (both p.a.) from Fluka Analytical (Steinheim, Germany). The APTS-labeled bi-antennary complex type glycans A1F, A2F, G0F, G1F, and G2F were obtained from Ludger (Oxfordshire, UK) (see structures in Supporting Information Fig. 1).

2.2 Sample preparation

BSA, *p*-TSA, and naphthalenesulfonic acid (NSA) were used for the method development. Stock solutions (10 g/L) of the sulfonic acids were obtained by dissolving each of them in water. Stock solutions were diluted with 0.2 M FAc, to obtain standard solutions. APTS-labeled glycans were dissolved in water (126 mg/L) to obtain a stock solution and further diluted with water.

2.3 SPE material

Strata X-AW and Strata XL-AW, Weak Anion Mixed Mode Materials (Phenomenex, Aschaffenburg, Germany) were used for the construction of the in-line SPE column. Strata X/XL-AW consists of the same porous polymer and differ only in the beads average diameter (ad) of 30 μm and 100 μm and the adsorption surface per gram (800 and 500 m^2/g, respectively). Other bead sizes were not available. This sorbent is a combination of weak anion exchanger and RP material, that is, containing repeating *N*-benzyl-ethylendiamine units in the side chain of the polymer. Due to the porous carbon-based polymer structure, the particles are soft and fragile. The material is designed for retention of acidic compounds with a pk_a-value less than 5. In order to remove interfering particle sizes, the Strata X-AW and the Strata XL-AW were sieved, using mesh widths of 37, 53, and 90 μm (LINKER Industrie Technik, Kassel, Germany).

2.4 CE

Separation was performed on a PA 800 plus Pharmaceutical Analysis System (Beckman Coulter, Brea, USA) and a PACE/MDQ instrument (Beckman Coulter, Brea, USA) with integrated UV-detectors. Fused silica capillaries (Polymicro Technologies, Phoenix Arizona, USA) with id of 30/50/75/100/150 μm and an od of 365 μm were used for the construction of in-line SPE-CE capillaries. Sulfonic acids were separated in a BGE of 0.2 M FAc, APTS-labeled glycans were separated using a BGE of 40 mM EACA and 130 mM acetic acid [54] and capillary segments neutrally coated with PVA, according to the protocol of Belder et al. [55]. For separation −20 kV (organic sulfonates) and −30 kV (APTS-labeled glycans) was applied to the capillary inlet.

2.5 In-line SPE-CE

An in-line-SPE-capillary consists of three individual parts of capillary: the SPE column (~0.4 cm) is placed between an inlet (≈11 cm) and a separation capillary (≈70 cm) with an id smaller than the SPE beads diameter. The capillaries are joined by two pieces of polyvinyl chloride tubing with an id of 190 μm. In order to fill the SPE column the particles were suspended in methanol and sucked into column by vacuum application. A straight cut of capillary ends is achieved using rotating diamond cutter (Agilent Technologies, Waldbronn, Germany).

Inline SPE-CE method was performed as follows: First, the capillary was rinsed with eluent, consisting of methanol/ammonia (30 w %) in the ratio of 95:5 v/v, in order to remove remnants from previous analyses. For equilibration the capillary is rinsed with the respective acidic BGE, which is similar to the off-line protocol for the SPE cartridges [56]. Afterwards, sample was loaded on the SPE column. Then, the capillary had to be refilled and equilibrated with the respective BGE again. Subsequently, the analytes were eluted using a short plug of eluent. After the elution plug was pushed over the SPE column the separation voltage was applied. Details and optimization of these steps are described in Section 3.2.

2.6 MS

MS was performed on the micrOTOFQ (Bruker Daltonik, Bremen, Germany), a quadrupole TOF (QTOF-MS) equipped with an ESI source. Coupling was performed using a coaxial sheath liquid interface from Agilent Technologies (Waldbronn, Germany); 0.025% triethylamine in isopropanol-water (1:1, v/v) was used as sheath liquid at a flow rate of 4 μL/ min. The ESI source was operated in the negative mode with a positive MS inlet (4 kV) and a grounded sprayer. The flow rate of the drying gas (nitrogen, >99.5%) was 4.0 L/min at a temperature of 170°C. Nebulizer gas (nitrogen, >99.5%) pressure was set to 0.2 bar. Spectra were acquired in a mass range of 400–2000 m/z at a repetition rate of 1 Hz. The instrument was calibrated daily by infusion of ES-TOF Tuning Mix (Agilent Technologies, New Castle, Delaware, USA). The software Data Analysis (Version 4.0 SP 2, Bruker Daltonik) was used for data evaluation.

3 Results and discussion

3.1 Construction of in-line SPE-CE capillary

In general, two different designs of SPE-beds were constructed as schematically depicted in Fig. 1: A) standard design with particle ad $< {}^1/_2 \times$ SPE columns id (type A) and B) the bead string design with particle ad slightly smaller than the id of the SPE column. All combination of bead and column diameter, which were evaluated are shown in Table 1.

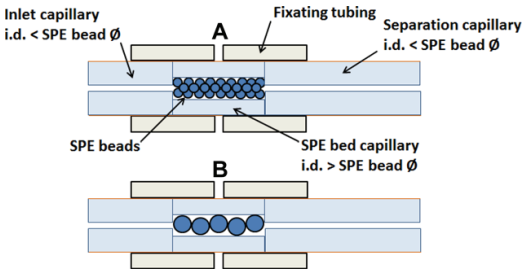

Figure 1. Two general designs of fritless in-line SPE-CE columns: (A) Inner diameter of the SPE column is much larger than the SPE particles' average diameter; (B) inner diameter of the SPE column is slightly larger than the SPE particles' diameter, leading to a single bead chain.

Table 1. Different geometries of in-line SPE columns used in this work

Particle diameter (μm)	SPE column id (μm)	Sep. capillary id (μm)	Construction type[a)]	Result
>37[b)]	50	30	B	Difficult to fill SPE column Often clogged by particles
>37[b)]	75	30	A	Easy to fill SPE column Often clogged by wedged particles
>37[b)]	100	30	A	Easy to fill SPE column Often clogged by wedged particles
>37[b)]	150	30	A	Easy to fill SPE column Often clogged by wedged particles
<90[c)]	100	50	B	Good overall performance, see text
<90[c)]	150	50	B	Easy to fill SPE column Low adsorption and re-elution ability

a) Compare Fig. 2
b) Sieved from mean of 33 μm.
c) >53 μm, sieved from mean of 100 μm.

Experiments showed, that the SPE columns of the common packed-bead-design (Type A) bear the risk of wedged beads, which led to clogging or formation of hollow spaces inside the SPE column using the here applied porous polymer Strata X/XL-AW material. In addition, particles hardly larger than the id of the separation capillary easily clogged the conjunction between the SPE column and the inlet/separation capillary.

Therefore, a bead string design (Type B) was applied using two different sizes (ad 100 and 33 μm). Also the bead string design capillaries with the small SPE beads (ad 33 μm) in 50 μm capillaries got frequently clogged, due to particles with similar id to the id of the separation capillary (30 μm).

Table 2. Final in-line SPE-CE method

Step	Solution	Volumes	Pressure (mBar)	Time (min)[a)]
1. Rinse	Eluent	0.5 V_{cap}	1000	0.79–1.28
2. Equilibrium	BGE	3 V_{cap}	1000	4.72–7.65
3. Load sample	Sample	0.6–6 V_{cap}	1000	0.98–15.85
4. Refill/rinse	BGE	3 V_{cap}	1000	4.72–7.65
5. Inject eluent	Eluent	0.02 V_{cap}	50	0.62–1.03
6. Push on SPE column	BGE	V_{inlet} + 0.5 (V_{SPE}–V_{plug})	500	0.37–0.62
7. Wait	BGE	–	–	1
8. Push from SPE column	BGE	0.22 V_{cap}–V_{inlet} + 0.5 (V_{SPE}–V_{plug})	50	2.50–4.23
9. Separation	BGE	–	−20 kV	20
Total	–	–	–	35.7–63.3

a) Times depending on the actual flow rate.

Due to the small particle diameter the filling process was in general more complicated and time-consuming in comparison to the following construction using particles of just under 90 μm ad. Thus, it was waved to optimize this device.

In contrast, the bead string (Type B) capillaries with particles of just under 90 μm (sieving particles with a mean of 100 μm using the 90 μm sieve) combined with separation capillaries of 50 μm id could be filled in seconds. Thereof, the bead string capillaries with 150 μm id SPE columns showed low adsorption and re-elution ability in comparison to 100 μm id SPE columns, which is expected to be caused by the much higher dead volume (i.e. factor of ≈3.5 larger). For this reason, bead string capillaries with particles just under 90 μm in 100 μm id SPE columns combined with a separation capillary of 50 μm were found to be best for the construction and, thus, used for further method development. Typically 45–55 particles were filled in a device of 4 mm length leading to an ad of 80–85 μm.

3.2 Flow rate determination

The flow rate is one of the most important parameters for method development of packed-bed inline SPE-CE capillaries. Unlike standard CE capillaries, using in-line SPE capillaries, the flow rate cannot be calculated theoretically using the Hagen–Poiseuille equation, since the effect of the SPE column (i.e. particle position, density of particles) on the flow conditions inside the capillary is unknown. Thus, empirical determination had to be performed: an elution plug was injected, pressure was applied and the flow rate calculated from the time of the UV-detector signal. For CE-MS application TSA (40 μg/mL) diluted in eluent was used to determine the flow rates. It turned out, that the correlation between flow rate and applied pressure were proportional up to 500 mbar (Supporting Information Fig. 2). From 1000 mbar, the flow rate did not increase proportionally to the applied pressure and approached a constant flow rate of about 2000 nL/min.

For a single SPE device the flow rate fluctuated. Over a period of one month, the flow rate (at 1000 mbar) was determined daily resulting in a RSD of 15.5 %. This is likely due to reorganization of the particles caused by pressure variations during analysis. Thus, the flow path changes, causing different flow conditions. For this reason the flow rate was determined daily.

3.3 In-line SPE-CE method

For the development and validation of the in-line SPE-CE method, UV-detection was used. The general steps of the in-line SPE-CE method were adapted from the off-line protocol from Jung et al. [56] and Bunz et al. [49]. However, an additional methanol rinsing step after sample loading was omitted here, since the methanol caused already elution of the analytes in the in-line SPE-CE approach (elution of 88–96%, data not shown). In Table 2 the detailed in-line SPE-CE method is listed, including all varied parameters. The method was systematically developed and optimized:

As a first step, the in-line SPE-CE capillary was rinsed with eluent (0.5 total capillary volumes), in order to remove possible remnants from previous analyses. For equilibration of the stationary phase, the capillary was rinsed with BGE (3 total capillary volumes).

The sample loading process may have influence on the analyte adsorption process. If the loading velocity is too high, it is possible that the analytes are not retained quantitatively. Therefore, the loading pressure was tested at 100, 500, and 100 mbar applying identical sample volumes. Since no significant increase of signal intensities was observed by using lower pressure settings, a pressure of 1000 mbar was used for a short loading time.

After loading, the capillary was rinsed and refilled with BGE, in order to flush nonretaining matrix out of the capillary. No elution was observed using the acidic BGE. Therefore, the capillary was thoroughly rinsed with BGE (three total capillary volumes).

Afterwards an elution plug with a volume of 35 nL was injected at 50 mbar. Higher and lower elution volumes were tested: lower volumes did not elute the analyte quantitatively; higher volumes did not enhance the recovery. The elution plug was pushed at 500 mbar to a position, where the center of the elution plug and the center of the SPE column match

(i.e. position calculated as shown in Table 2). The position was maintained over 1 min to allow the eluent to soak into SPE beads and to desorb the analytes. Without waiting time the analytes could not be fully eluted. No significant improvement of the signal intensities was obtained using a waiting time of 5 min.

Before applying voltage, the elution plug has to be placed behind the SPE bed, as otherwise current breakdown occurs, probably caused by bubble formation when the low conductivity elution plug resides on the SPE column. Therefore, the elution plug has to be pushed into the capillary, before the separation voltage was applied. The pushing step was determined empirically: First, the elution plug was pushed far into the capillary (0.6 min at 1000 mbar), in order to ensure that the elution plug definitely did not reside on the SPE column. The pushing time was decreased, until the current showed abnormal behavior or broke down. Below a pushing time of 0.3 min at 1000 mbar, the current began to fluctuate and partly broke down. This corresponded to a position about 6 cm after the SPE column. This position was transferable to other in-line SPE-CE capillaries.

The final in-line SPE-CE method parameters are listed in Table 2. The resulting performance is shown in Fig. 2 based on the analysis of BSA, TSA, and NSA. The achieved resolution R is similar to the standard CE-UV analysis without SPE (data not shown).

3.4 Method validation

Repeatability of the optimized inline-SPE-CE-UV method was determined injecting 15 times in row the sulfonic acid standard at a concentration of 1 μg/mL each. This corresponds to a total amount of 1 ng respectively, by using a sample loading volume of 1 μL. Acceptable RSD values between 3–7% for the peak areas and 2–5% for the peak intensities were achieved.

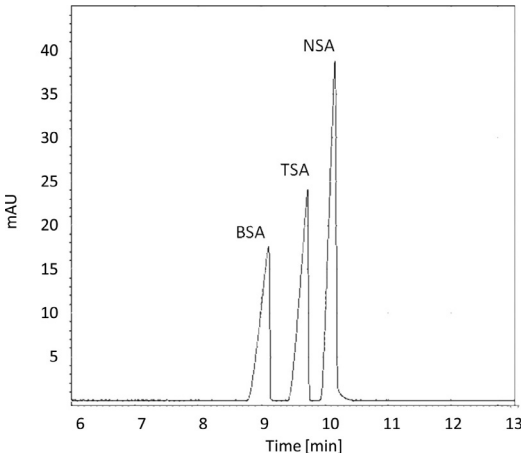

Figure 2. Electropherogram of BSA, TSA (at 190 nm), and NSA (at 224 nm) using an in-line SPE-CE-UV system. The analysis was performed applying the optimized method (see text) injecting 1 μL of standard mix (1 ng each on column)

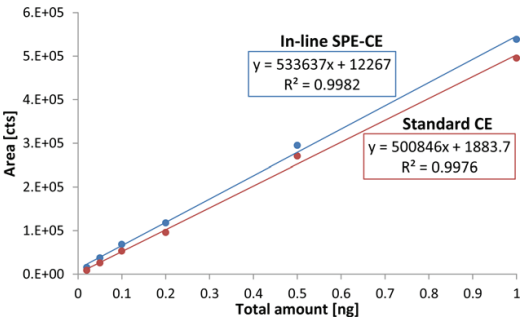

Figure 3. Comparison of the in-line SPE-CE and a standard CE system calculating identical injection amounts. The mean values of the peak areas of each concentration level (three repetitions each) are shown for BSA.

Table 3. LODs in in-line SPE-CE and standard CE for three organic sulfonates

Substance	In-line SPE-CE LOD (ppb)	Standard CE LOD (ppm)	Enrichment factor
BSA	2.0	0.9	450
NSA	1.0	0.9	900
TSA	0.3	0.3	1000

The elution efficiency was tested by running the method without rinsing equilibration and sample loading (steps 1–3 Table 2) directly after the standard method. All together six experiments were performed on two in-line SPE capillaries. Both capillaries delivered similar results (data not shown). A mean of 98.3% (no value <96.7%) was eluted in the first of the two elution steps. This value is acceptable, especially considering that the purpose of the in-line SPE-CE system is to enrich analytes by a large factor.

For comparison of the calibration curve a total amount of analytes of 0.02–1 ng were injected on a standard CE system and the inline SPE-CE, respectively. Both systems used a separation capillary of about 70 cm in length. Six concentration levels in a range of 2–100 ng/mL were analyzed by in-line SPE-CE using a sample loading volume of 10 μL and six calibration standards in a range of 1.5–75.5 μg/mL by standard CE using a calculated injection volume of 13.24 nL. Both, the in-line SPE-CE system and standard CE system, showed linearity in the working range for all three analytes and similar sensitivity for same total sample amounts (see data of BSA in Fig. 3). Assuming a correct injection calculation a mean recovery of 106% can be determined based on the slopes of the calibration line. The LODs of both systems were estimated, using the S/N ratios of the lowest concentration level. Enrichment factors between 450 and 1000 (Table 3) were calculated. The LODs of the in-line SPE-CE system, and therefore the

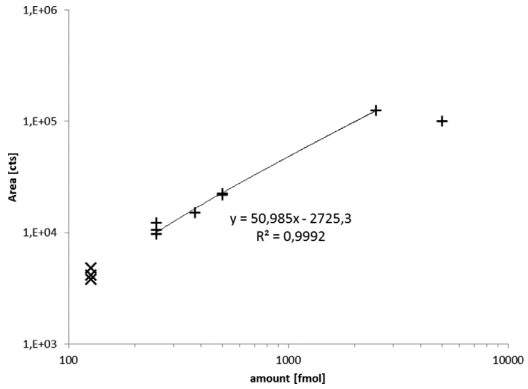

Figure 4. Comparison of in-line SPE-CE-MS (+) and standard CE-MS (x) for the analyses of the glycan A1F. Extrapolation was performed for in-line SPE-CE-MS data up to 2500 fmol.

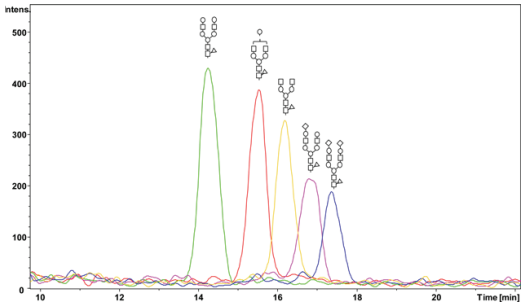

Figure 5. Extracted ion electropherograms of five APTS-labeled glycans (double and triple charged species). The separation was carried out in a PVA-coated capillary using a BGE of 40 mM EACA and 130 mM acetic acid at −30 kV.

enrichment factors, could be further improved by increasing the sample loading volume.

The bed capacity was determined by three repetitive breakthrough experiments for each analyte using a concentration of 20 ng/μL and an injection pressure of 1000 mbar. The mean bed capacity was between 123 and 512 ng for BSA and NSA, respectively. These values are absolutely sufficient considering the fact that a 1 min injection of a 1 ng/μL solution corresponds to an amount of 10 ng. Such a 1 ng/μL solution can be measured directly by CE-UV without any preconcentration.

Using the complete proposed protocol (including daily flow rate determination) several in-line SPE-CE devices were used successfully over several months. Thus, the in-line SPE-CE system is not only easy to construct but also stable to operate over long time. If in rare cases the system instability occurs in terms of current breakdown, the flow rate is determined again and the method functionality is restored.

3.5 APTS-labeled glycans

The aim of the following experiments was the successful enrichment of APTS-labeled glycans using the in-line SPE-CE, followed by TOF-MS detection. An EACA-based BGE and a PVA-coated capillary were used (for details see Section 2.4).

APTS-labeled A1F glycan (see structure in Supporting Information Fig. 1) was chosen for calibration. The linearity was determined in a range of 250–5000 fmol applying a 50 and 500 nM standard and loading volumes of 5, 7.5, and 10 μL. Good linearity was determined up to 2500 fmol. At 5000 fmol the signal intensity decreased and deviate from the linear calibration curve (using a double logarithmic scale, see Fig. 4). Though this linear range up to 2500 fmol is sufficient for CE-MS characterization, the bed capacity is expected to be larger (compare BSA, TSA, NSA). However, the sample contains a 30-fold excess of the derivatizating reagent APTS.

In order to verify the comparability of the in-line SPE-CE system with a standard CE system, a 10 μM solution of APTS-labeled A1F was analyzed with standard CE-TOF-MS. A total injection volume of 12.6 nL containing a total amount of 126 fmol standard was injected at 50 mbar over 12 s. The analysis was repeated three times. The determined peak areas correspond to the expected values based on the linear regression of the in-line SPE-CE system, as depicted in Fig. 4. A 50 nM standard analyzed by in-line SPE-CE-MS delivered equivalent peak areas as a 40 μM standard using standard CE-MS instrumentation (data not shown). This corresponds to an enrichment factor of 800.

Subsequently, a glycan mixture containing five different bi-antennary complex type *N*-glycans (A1F, A2F, G0F, G1F, and G2F) was successfully analyzed using the inline SPE-CE-TOF-MS system (Fig. 5). All five glycans are separated, though not baseline separated. The resolution is similar to the standard CE-MS analysis without SPE (data not shown), when using the same capillary dimension. However, the capillary length needs to be extended in order to separate structural isomers as G1F and G1F′, which was shown recently using the same separation system [54].

4 Concluding remarks

The presented novel in-line SPE-CE technique using a bead string design provides easy and well reproducible construction as well as high recovery rates. The used mixed-mode phase is applicable to both, organic sulfonates as well as APTS-labeled glycans. A considerable improvement of the detection limit could be achieved, with enrichment factors between 450 and 1000 for three different organic sulfonates as well as the APTS-labeled A1F glycan standard applying a sample volume of 10 μL. These enrichment factors can even be further improved by increasing the sample loading volume. However, the overall run time of typically 45 min would be further extended. The sensitivity of the new developed in-line SPE-CE method was demonstrated by LOD concentrations for organic sulfonates in the sub-ppb range. The in-line

SPE-CE works in general stable and reproducible. Variations in the flow rate might occur and need to be determined regularly. A more uniform size distribution of the applied SPE particles (using other sieves) is expected to improve flow rate stability.

The enrichment and separation of a mixture of five APTS-labeled glycans demonstrate the potential of the presented technique. However, the glycan method has to be investigated further, in order to improve the separation and to get a better understanding of the bed capacity with respect to an excess of labeling reagents.

The authors have declared no conflict of interest.

5 References

[1] Chen, Y., Lü, W., Chen, X., Teng, M., *Cent. Eur. J. Chem.* 2012, *10*, 611–638.

[2] Malá, Z., Gebauer, P., Boček, P., *Electrophoresis* 2013, *34*, 19–28.

[3] Kazarian, A. A., Hilder, E. F., Breadmore, M. C., *J. Sep. Sci.* 2011, *34*, 2800–2821.

[4] Neusüß, C., Pelzing, M., Macht, M., *Electrophoresis* 2002, *23*, 3149–3159.

[5] Breadmore, M. C., Boyce, M. C., Macka, M., Avdalovic, N., Haddad, P. R., *Analyst* 2000, *125*, 799–802.

[6] Hutchinson, J. P., Macka, M., Avdalovic, N., Haddad, P. R., *J. Chromatogr. A* 2004, *1039*, 187–192.

[7] Zhang, S., Macka, M., Haddad, P. R., *Electrophoresis* 2006, *27*, 1069–1077.

[8] Breadmore, M. C., *Anal. Chim. Acta* 2006, *556*, 121–126.

[9] Chaisuwan, P., Nacapricha, D., Wilairat, P., Jiang, Z., William Smith, N., *Electrophoresis* 2008, *29*, 4008–4016.

[10] Thabano, J. R. E., Breadmore, M. C., Hutchinson, J. P., Johns, C., Haddad, P. R., *J. Chromatogr. A* 2007, *1175*, 117–126.

[11] Vanderschaeghe, D., Szekrenyes, A., Wenz, C., Gassmann, M., Naik, N., Bynum, M., Yin, H., Delanghe, J., Guttman, A., Callewaert, N., *Anal. Chem.* 2010, *82*, 7408–7415.

[12] Tomlinson, A. J., Guzman, N. A., Naylor, S., *J Capill. Electrophor.* 1995, *2*, 247–266.

[13] Pelzing, M., Neusüß, C., *Electrophoresis* 2005, *26*, 2717–2728.

[14] Tomlinson, A. J., Benson, L. M., Jameson, S., Johnson, D. H., Naylor, S., *J. Am. Soc. Mass Spectrom.* 1997, *8*, 15–24.

[15] Tomlinson, A. J., Benson, L. M., Guzman, N. A., Naylor, S., *JCA* 1996, *744*.

[16] Naylor, S., Ji, Q., Johnson, K. L., Tomlinson, A. J., Kieper, W. C., Jameson, S. C., *Electrophoresis* 1998, *19*, 2207–2212.

[17] Li, J., Thibault, P., Martin, A., Richards, J. C., Wakarchuk, W. W., van der Wilp, W., *J. Chromatogr. A* 1998, *817*, 325–336.

[18] Botello, I., Borrull, F., Aguilar, C., Calull, M., *Electrophoresis* 2012, *33*, 528–535.

[19] Botello, I., Borrull, F., Calull, M., Aguilar, C., Somsen, G., Jong, G., *Anal. Bioanal. Chem.* 2012, *403*, 777–784.

[20] Lara, F. J., García-Campaña, A. M., Alés-Barrero, F., Bosque-Sendra, J. M., *Electrophoresis* 2008, *29*, 2117–2125.

[21] Macià, A., Borrull, F., Calull, M., Benavente, F., Hernández, E., Sanz-Nebot, V., Barbosa, J., Aguilar, C., *J. Sep. Sci.* 2008, *31*, 872–880.

[22] Maijó, I., Borrull, F., Aguilar, C., Calull, M., *Electrophoresis* 2013, *34*, 363–373.

[23] Maijo, I., Borrull, F., Calull, M., Aguilar, C., *Electrophoresis* 2011, *32*, 2114–2122.

[24] Maijó, I., Fontanals, N., Borrull, F., Neusüß, C., Calull, M., Aguilar, C., *Electrophoresis* 2013, *34*, 374–382.

[25] Saavedra, L., Maeso, N., Cifuentes, A., Barbas, C., *J. Pharm. Biomed. Anal.* 2007, *44*, 471–476.

[26] Tak, Y. H., Toraño, J. S., Somsen, G. W., de Jong, G. J., *J. Chromatogr. A* 2012, *1267*, 138–143.

[27] Viberg, P., Wahlund, K.-G., Skog, K., *J. Chromatogr. A* 2006, *1133*, 347–352.

[28] Lara, F. J., García-Campaña, A. M., Neusüss, C., Alés-Barrero, F., *J. Chromatogr. A* 2009, *1216*, 3372–3379.

[29] Tong, W., Link, A., Eng, J. K., Yates III, J. R., *Anal. Chem.* 1999, *71*, 2270–2278.

[30] Guzman, N. A., Stubbs, R. J., *Electrophoresis* 2001, *22*, 3602–3628.

[31] Figeys, D., Ducret, A., Aebersold, R., *JCA* 1997, *763*, 295–306.

[32] Figeys, D., Corthals, G. L., Gallis, B., Goodlett, D. R., Ducret, A., Corson, M. A., Aebersold, R., *Anal. Chem.* 1999, *71*, 2279–2287.

[33] Bateman, K. P., White, R. L., Thibault, P., *J. Mass Spectrom.* 1998, *33*, 1109–1123.

[34] Swartz, M. E., Merion, M., *J. Chromatogr. A* 1993, *632*, 209–213.

[35] Zhang, P., Xu, G., Xiong, J., Zheng, Y., Shi, X., Yang, Q., Wei, F., *J. Sep. Sci.* 2003, *26*, 1527–1532.

[36] Lara, F. J., García-Campaña, A. M., Neusüss, C., Alés-Barrero, F., *J. Chromatogr. A* 2009, *1216*, 3372–3379.

[37] Lee, W.-H., Wang, C.-W., Her, G.-R., *Rapid Commun. Mass Spectrom.* 2011, *25*, 2124–2130.

[38] Lee, W.-H., Her, G.-R., *Electrophoresis* 2009, *30*, 1675–1683.

[39] Medina-Casanellas, S., Benavente, F., Barbosa, J., Sanz-Nebot, V., *Electrophoresis* 2011, *32*, 1750–1759.

[40] Schaller, D., Hilder, E. F., Haddad, P. R., *Anal. Chim. Acta* 2006, *556*, 104–111.

[41] Hernández, E., Benavente, F., Sanz-Nebot, V., Barbosa, J., *Electrophoresis* 2008, *29*, 3366–3376.

[42] Almeda, S., Arce, L., Benavente, F., Sanz-Nebot, V., Barbosa, J., Valcárcel, M., *Anal. Bioanal. Chem.* 2009, *394*, 609–615.

[43] Ramautar, R., de Jong, G. J., Somsen, G. W., *Electrophoresis* 2012, *33*, 243–250.

[44] Ramautar, R., Somsen, G. W., de Jong, G. J., *Electrophoresis* 2010, *31*, 44–54.

[45] Saavedra, L., Barbas, C., *J. Biochem. Biophys. Methods* 2007, *70*, 289–297.

[46] Siren, H., Seppanen-Laakso, T., Oresic, M., *J. Chromatogr. B* 2008, *871*, 375–382.

[47] Molina-Mayo, C., Hernandez-Borges, J., Borges-Miquel, T. M., Rodriguez-Delgado, M. A., *J. Chromatogr. A* 2007, *1150*, 348–355.

[48] Ge, L., Yong, J. W. H., Tan, S. N., Yang, X. H., Ong, E. S., *J. Chromatogr. A* 2006, *1133*, 322–331.

[49] Bunz, S.-C., Weinmann, W., Neusüß, C., *Electrophoresis* 2010, *31*, 1274–1281.

[50] Bringmann, G., Kajahn, I., Neusüß, C., Pelzing, M., Laug, S., Unger, M., Holzgrabe, U., *Electrophoresis* 2005, *26*, 1513–1522.

[51] Gennaro, L. A., Salas-Solano, O., *Anal. Chem.* 2008, *80*, 3838–3845.

[52] Schwarzer, J., Rapp, E., Reichl, U., *Electrophoresis* 2008, *29*, 4203–4214.

[53] Ruhaak, L. R., Hennig, R., Huhn, C., Borowiak, M., Dolhain, R. J. E. M., Deelder, A. M., Rapp, E., Wuhrer, M., *J. Proteome Res.* 2010, *9*, 6655–6664.

[54] Bunz, S.-C., Rapp, E., Neusüß, C., *Anal. Chem.* 2013, *85*, 10218–10224.

[55] Belder, D., Deege, A., Husmann, H., Kohler, F., Ludwig, M., *Electrophoresis* 2001, *22*, 3813–3818.

[56] Jung, B., Caslavska, J., Thormann, W., *J. Chromatogr. A* 2008, *1206*, 26–32.

Chapter 3

Discussion

CSE-LIF systems are widely used for routine glycan analysis [55, 78-81]. CE-LIF provides high resolving power combined with highly sensitive detection. The technique is perfectly suited for glycan pattern analysis in complex samples. Two powerful CSE-LIF systems for the analysis of APTS-labeled glycans are based on commercial high viscosity electrolytes: the glycan gel separation buffer (further referred to as "GEL1") from Beckman Coulter (Brea, US) and the Pop-7 gel (further referred to as "GEL2") from Applied Biosystems (Life Technologies, UK). Their linear polymers provide an additional sieving effect, whereby size and shape alter the electrophoretic mobility of the charged analytes. Both systems provide high separation efficiency including separation of isomeric glycans with minor structural diversity. However, firsthand identification of unknown species is hardly possible. As an example, Fig. 3 depicts a CSE-LIF electropherogram of the APTS-labeled glycan pool derived from a recombinant fusion protein. The analysis was performed using the commercial GEL2 as sieving electrolyte and a DNA-sequencer system (method published by Ruhaak et al. [80]). The high separation power of CSE using GEL2 is demonstrated; however, 4 of the 15 LIF-signals could not be identified (by glycan standards or mobility databases).

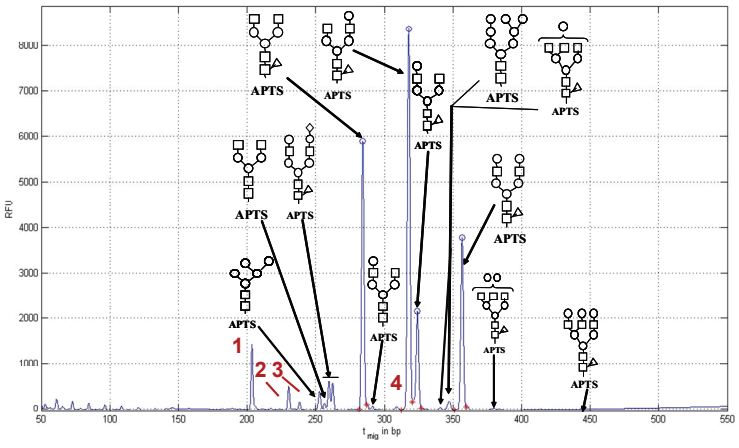

Fig. 3: CSE-LIF electropherogram of APTS-labeled glycans derived from a recombinant fusion protein. Unknown LIF-signals are annotated by red numbers (1-4). The analysis was performed using GEL2 in a fused silica capillary of 50cm length at +30 kV (method of Ruhaak et el.[80])

CE-MS is a powerful tool combining the high separation efficiency of CE and the capability of glycan mass detection by MS. Although CE is widely used in glycan analysis, CE-MS methods are rare [19, 59, 75-77] (manuscript 1). The polymeric electrolytes of CSE are not compatible with MS detection; thus, novel CE-MS compatible electrolytes are required. Only few methods for APTS-labeled glycans [61, 82-85] have been published so far. A reliable comparison in terms of separation efficiencies of the published systems is rather difficult as different types of glycans were used (e.g. O-acetylated glycans from fish serum analyzed by Jayo et al. [84]). Only Gennaro et al. presented a highly resolving separation by CE-MS for APTS-labeled complex type N-glycans derived from antibodies [82].

This thesis proposes two highly efficient CE-MS methods for the analysis of APTS-labeled glycans, comparable to CSE-LIF approaches, to allow the identification of unknown LIF signals: i) a robust system based on an alkaline BGE with cathodic detection (manuscript 2) and ii) an acidic CE-MS system at reverse polarity with a migration order analogous to standard CSE-LIF approaches (manuscript 3). Both methods were optimized and validated in terms of sensitivity, separation efficiency and reproducibility for the glycan analysis of biopharmaceutical products. Two glycan isomers (G1F and G1F': two bi-antennary, core-fucosylated complex type N-glycans with one galactose residue linked to either of the two antennae) were used to examine the system's resolving power. Additionally, the capability to identify peaks from standard CSE-LIF systems, i.e. mobilities of various APTS-labeled glycans, was examined. Therefore, the acidic and the alkaline CE-MS methods developed in this thesis and two CSE-LIF methods (using GEL1 and GEL2) were compared with respect to glycan mobilities. A full signal assignment of a biopharmaceutical sample is presented (manuscript 2).

Finally, an in-line SPE-CE-MS setup was developed to increase the sensitivity in terms of minimal sample concentration, to enable the detection of sample in the low femto-molar (fM)-range as analyzed in many CE-LIF approaches.

In the following paragraphs the alkaline and the acidic CE-MS method as well as glycan identification and peak assignment will be discussed. Furthermore, the important aspects of in-line SPE-CE-MS setup are addressed.

3.1 CE-MS Analysis: Alkaline Method

3.1.1 Method Development and Validation

The high performance of the previously mentioned method by Gennaro et al. was difficult to reproduce due to its high pressure sensitivity. In contrast, the alkaline CE-MS method for charged native glycans, previously published by our group [86], was robust for CE-MS applications due to its strong EOF. However, the performance for APTS-labeled glycans is not satisfying (it shows broad peaks and low separation efficiency). Thus, the method was adapted for the use of APTS-labeled glycans resulting in a BGE of 0.7 M ammonia, 0.1 M ε-aminocaproic acid (EACA) in 70 % (v/v) methanol/water and SL of 50 % (v/v) 2-propanol/water. The separation was performed in fused silica capillaries of 80-90 cm length with cathodic detection (+30 kV). The final method allows the simultaneous separation of APTS-labeled glycans as well as charged native glycans. Therefore, the labeling and ionization efficiency could be determined and was used to quantify glycans after the labeling process. The migration order is in general reversed compared to the standard CSE approaches.

Method validation was performed based on four different APTS-labeled bi-antennary-complex-type glycan standards (G0F, G1F, G1F' and G2F, see structures Fig. 1), with good structural isomer separation. The method's strong EOF facilitated the coupling of CE-MS and allowed a reproducible performance with good migration time precision, i.e. RSD of 4.7%, and appropriate resolution (R) of two isomeric G1F peaks based on the full-width-half-maximum (FWHM) (average R for G1F and G1F': R = 1.1). Linearity was determined by an external standard calibration method in a concentration range of 1.5-48 µM with a regression coefficient of $r^2 = 0.9988$ (manuscript 2).

3.1.2 Application to Biopharmaceutical Products

The alkaline BGE system was applied to identify and characterize glycan species of different glycosylated biopharmaceutical products like therapeutic antibodies, fusion proteins or cytokines, previously analyzed by CSE-LIF. By the new method additional glycan species were identified. The following example presents the identification results of APTS-labeled glycans derived from a recombinant fusion protein (CSE-LIF results given in Fig. 3). Glycan identification was performed according to paragraph 3.3. The extracted ion electropherograms (EIEs) of all analyzed species are listed in Fig. 4. In this way 11 of 12 previously identified glycan species as marked in Fig. 3 were confirmed and all four unknown species, annotated by red numbers (1-4), were characterized. Due to the

low amount of sample, the last CSE-LIF signal of a tri-antennary, complex-type glycan was not detectable by MS.

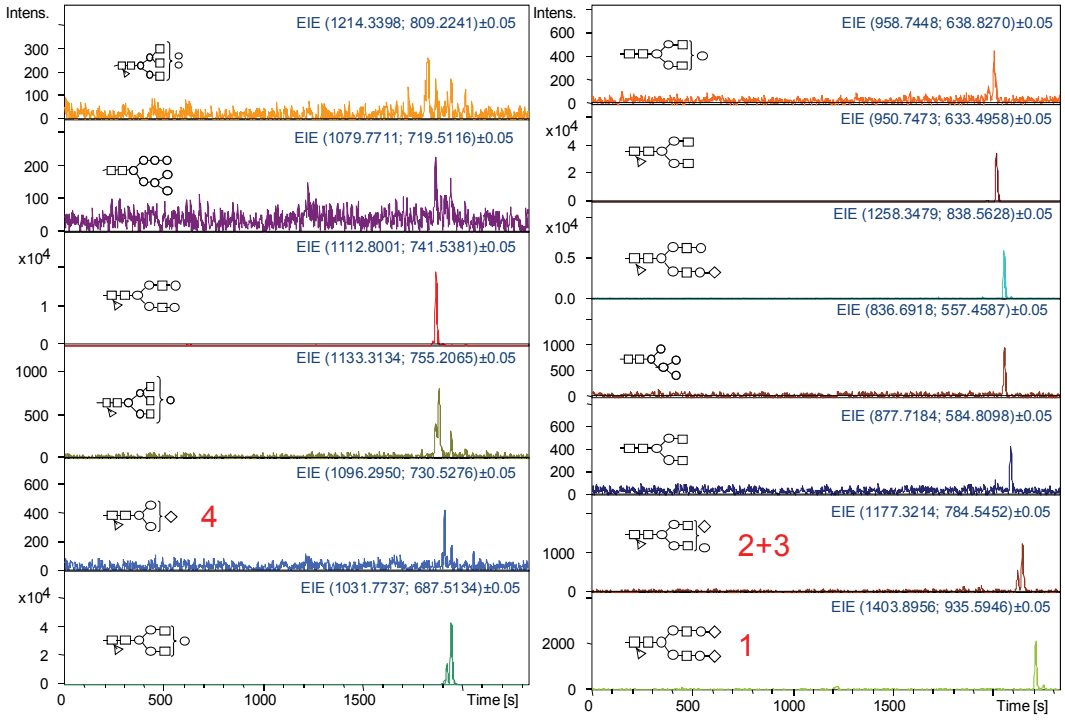

Fig. 4: EIEs of all identified glycan species of a recombinant fusion protein applying the proposed alkaline CE-MS method (BGE as discussed in text, capillary length of 90 cm,). The previously unknown glycan species are annotated according to Fig. 3 in red numbers (1-4). Therefore, the m/z values of the first isotope of the doubly and triply charged glycan are summed up to one trace as annotated individually.

In addition the characterized glycan compositions were verified comparing the relative mobilities in CSE-LIF to the CE-MS system (see manuscript 3). In this way, the identified glycans were clearly assigned to CE-LIF signals, despite the generally reversed migration order and the relatively shifted glycan mobilities.

3.2 CE-MS Analysis: Acidic Method

One of the first and most promising methods for CE-MS analysis of APTS-labeled glycans has been published by Gennaro et al. [82]. They achieved high separation efficiency for glycan structural isomers. Therefore, the method has shown potential to identify unknown glycan signals in standard CSE-LIF approaches. However, the published performance of this method has turned out to be difficult to reproduce. The main reason is most likely the sensitivity to pressure changes (i.e. by siphoning effects or

nebulizer gas impact), which can occur for CE-MS systems with negligible EOF (as already mentioned by Gennaro et al.). Alternative parameters with impact on separation efficiency are the BGE and SL compositions, the capillary coating as well as temperature. Therefore these parameters as well as the neutral capillary coating were systematically studied and optimized to develop a reproducible, highly efficient CE-MS method for the analysis of APTS-labeled glycans.

3.2.1 Method Development: BGE Composition and Temperature Influence

Initially different acidic BGE systems based on formiate (0.2 M and 0.8 M) and acetate (0.2 M - 0.5 M) were tested; however, compared to the data presented by Gennaro et al. these systems provide broad peak shapes and less resolving power. Consequently, the BGE was optimized varying the parameters used by Gennaro et al. (40 mM EACA at pH 4.5) i.e. the EACA concentration and the ammonia concentration in the BGE (and thus, pH). Finally the optimal BGE was found to be 40 mM EACA at a pH of 4.0 (131 mM acetic acid). The experiments were performed using a capillary of 80 cm length, this length was found to be suitable for efficient separation of structural glycan isomers in complex samples. In this setup, the method provides higher resolution and better peak shapes compared to the previously given parameters indicated by Gennaro et al..

Additionally, the influence of ambient temperature on the CE separation was studied and varied systematically (between 15°C and 27.5 °C) in a temperature controlled CE-LIF setup. The migration times of APTS-labeled glycans shifted strongly, i.e. about 2 min per 2.5 °C. Thus, to avoid changes in migration times the ambient temperature should be kept constant.

3.2.2 Sheath Liquid

In capillary systems with little or no EOF, as the here proposed acidic CE-MS method, the electromigration of ionic compounds is the only transport mechanism and electroneutrality should be maintained [87]. The substitution of exiting counter migrating ions may additionally affect separation efficiency, since potential ionic interaction with the analyte may have an influence on separation. Experiments with and without a feed of counter migrating ions demonstrated: without substitution the separation efficiency decreases, i.e. separation of structural glycan isomers is no longer possible. In CE-MS systems no outlet vial with counter ions is attached, here the SL is the only ion source. Ions with the same charge will automatically migrate into the separation capillary to maintain the inner electroneutrality. In this way the separation efficiency might be influenced as well, since moving ionic boundaries may be created. Ionic boundaries are

formed when the SL ions defer from BGE ions (i.e. different mobilities). The boundaries between BGE and the altered electrolyte are either diffuse or sharp, dependent on the conductivity and mobility differences in both zones [88].

Under the proposed conditions at pH 4.0 EACA is positively charged (pK_a: 4.4) and migrates out of the capillary on the inlet side. Neutral or acidic SL compositions cause a current decrease in the acidic EACA-based system, since the EACA ions exit the capillary and the BGE gets depleted. Therefore the addition of EACA to the SL in concentrations between 12 mM and 40 mM EACA was tested in CE-MS. However, under these conditions the peaks became broad and noisy. Thus, SL compositions with alternative basic additives such as aniline, ammonia and triethylamine (TEA) were tested to compensate the loss of cations. In the proposed system the positively charged amines enter from the SL into the capillary and migrate towards the inlet. Thereof, TEA was found to be best in terms of spray behavior and moderate current increase during separation (manuscript 3). The conductivity of the newly formed BGE differs significantly from the original BGE, thus a constantly increasing current profile is generated.

Replacing isopropanol by alternative organic solvents, as methanol or acetonitrile, decreased the signal intensities. Thus, the optimal SL composition was found to be 0.025% TEA in 50% (v/v) 2-propanol/water. The influence of the SL composition on separation efficiency was tested by replacing the BGE (40 mM EACA, 131 mM acetic acid) by SL in a CE-LIF experiment (results are shown in Fig. 5).

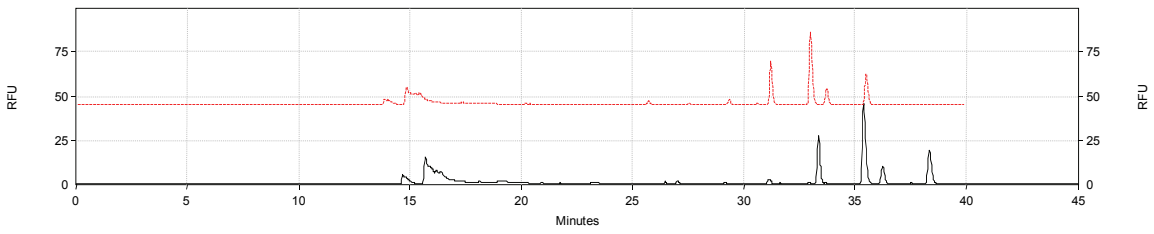

Fig. 5: Comparison of CE-LIF separation using the proposed acidic BGE and an outlet vial filled with either the same acidic BGE (red) or the optimized SL, as described in the text (black). The analysis was performed in row on a Beckman Coulter PA800+ instrument using the internal LIF detector (including an argon-ion laser source, excitation at 488nm, emission detected > 520 nm).

Compared to the setup with BGE in the outlet vial, the migration time was reduced and the separation efficiency was maintained when SL was provided in the outlet vial. The only disadvantage using the proposed SL is the reduced sensitivity (i.e. factor of 3) for analytes compared to the neutral SL composition (i.e. 50% (v/v) 2-propanol/water) (manuscript 3).

3.2.3 Influence of Electrospray and Pressure Effects

Beside the optimization of standard CE parameters, system affecting parameters were evaluated. In preliminary studies differences in separation efficiency and peak shape between CE-LIF and CE-MS within the same electrolyte system were observed. Therefore the influence of the electrospray process on the applied system was tested using an in-line CE-LIF-MS setup.

The in-line CE-LIF-MS setup allows the combination of the highly sensitive LIF detection with the high resolution and accurate mass detection by MS. A CE-LIF-MS setup, developed in our group and recently presented by Huhn et al. [89] was applied. The detectors were positioned as close as possible to each other, to minimize the changes in migration time and peak resolution. Therefore, a homemade capillary holder was fixed right on top of the applied sheath flow sprayer. In this way a minimized distance of about 12 cm between the detection units was obtained (more technical data are given in manuscript 2). The analytes' signals may differ in intensity between LIF and MS, as the responses of the analytes change due to the different detection techniques. However, the in-line assembly of both detectors enables the direct peak assignment of LIF to MS independently from signal intensities. Therefore the setup enables a simplified identification of glycans.

In this work in-line CE-LIF-MS detection was applied to evaluate the impact of the electrospray event on separation. The comparison of LIF and MS analysis applying the proposed acidic CE-MS method is shown in Fig. 6. The EIEs of four main glycan species of a recombinant fusion protein (G0F, G1F, G1F' and G2F, see structures Fig. 1), are depicted in one electropherogram together with the corresponding LIF-trace.

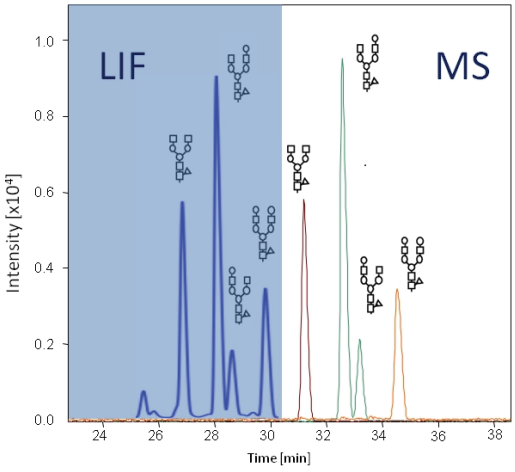

Fig. 6: In-line CE-LIF-MS setup applied to the acidic glycan analysis of a recombinant fusion protein. The LIF electropherogram of the four main glycan species (blue) was overlaid to the appropriate EIE generated by MS (using the doubly, triply and four-fold charged glycan masses)

The minor influence of the electrospray process on the systems was demonstrated, as the signal peak width and resolution is comparable to the in-line LIF detection. Additional CE-LIF-experiments showed good results with SL added to outlet vial as demonstrated in paragraph 3.2.2, Fig. 5. Hence, a direct impact of the electrospray process on the separation efficiency can be precluded.

Subsequent experiments confirmed the pressure sensitivity of the proposed system due to the low inherent flow and the missing pressure control at the capillary outlet. Thus, even small pressure imbalances as siphoning occurrence or potential suction effects (ESI nebulizer) affected the separation significantly (see manuscript 3). However, these effects could be reduced e.g. by setting the nebulizer gas flow as low as possible to maintain a stable electrospray signal or adjusting the capillary inlet height to avoid siphoning. Furthermore the ESI-sprayer condition (i.e. corrosion effects or other damages that might occur at the sprayer tip) may affect the nebulizing process and thus also the resulting suction. In the work presented here, separation efficiencies appropriate for the identification of glycan species detected by CE-LIF (average resolution of two isomeric G1F peaks: $R = 1.1$) were obtained, when the described pressure influencing parameters were adjusted properly. However, the separation efficiency is still reduced compared to the CE-LIF detection with SL in the outlet vial (resolution of G1F/G1F': $R = 2.5$, compare Fig. 5 to Fig. 7) – probably due to remaining pressure effects. The system can be counterbalanced by applying pressure in the low mbar range to the capillary inlet to increase separation efficiency. Nevertheless, system balancing by pressure was not followed in this work since the resolution was sufficient to assign the CE-MS signals to the applied CSE-LIF approach of Ruhaak (resolution of G1F/G1F': $R = 2.2$). Furthermore, applying pressure would prolong the migration times and the required precise adjustment of vacuum in the low mbar range is not supported by all type of CE instruments (e.g. PACE/MDQ, Beckman Coulter), thus, method transfers might become more complicated.

3.2.4 Capillary Coating

The application of anodic MS-detection requires EOF prevention, since any EOF would be directed towards the capillary entrance, cause SL or air intake and thus would disturb the separation. The EOF can be suppressed by using either a BGE with a $pH \leq 2$ (most silanol groups on the capillary surface are protonated under these conditions) or alternatively neutral capillary coatings. As for the proposed acidic BGE system at pH 4.0 (manuscript 2), different static neutral coatings, such as PVA, PAA and "Guarant" as well as a neutral dynamic coating based on soluble PAA ("LN", low normal) were tested. The

capillary coatings showed similar performances when using the same capillary lengths. However, permanent coatings are preferred for routine analysis, since no recoating is necessary. Hence a commercial PVA-coated capillary was chosen due to its performance and availability at capillary lengths up to 125 cm. Additionally, a homemade PVA-coating procedure (adapted from Belder et al. [90]) was implemented alternatively to the commercial PVA capillaries.

The comparability of migration times between different capillaries (two commercial and four homemade capillaries) was evaluated exemplarily with the two structural isomers of G1F. A relative standard deviation (RSD) of 4% between all capillaries demonstrated the reliability of the coating procedure and the comparability of homemade and commercial capillaries. The robustness of the capillary coatings was examined by CE-LIF (instead of CE-MS, to preclude any pressure influences due to missing pressure control at the capillary outlet). Fig. 7 shows the overlay of two electropherograms of APTS-labeled glycans released from a recombinant fusion protein. The samples were analyzed 36-times on a homemade PVA-capillary of 90 cm effective length. The shift in migration time between the two runs was caused by the varying ambient temperature.

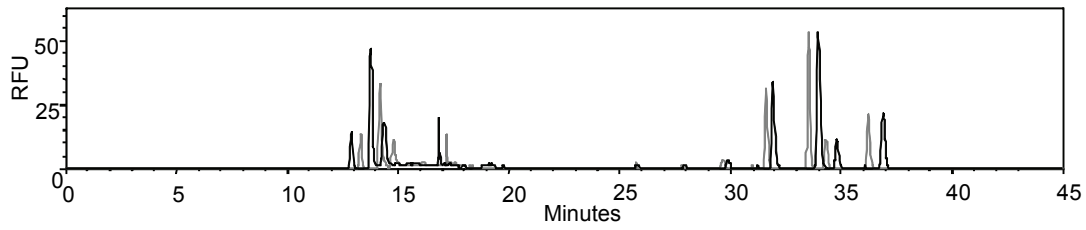

Fig. 7: Overlay of CE-LIF electropherograms of an APTS-labeled glycan sample from a recombinant fusion protein (run 1 and 36). Analysis was performed on a PVA capillary of 90 cm effective length using the optimized BGE in the inlet vial and SL in the outlet vial.

It should be noted that dry storage conditions are required for the ability to reuse the PVA capillary after longer storage time (more than 3-4 days). Hence, the water-cleaned capillary was flushed with air or nitrogen at a pressure of 5 bar for 20 min to remove any remaining water drops from the capillary surface. Furthermore, a mixture of 50% (v/v) methanol/water can be used to support the drying process, without impacting the coating layer.

3.2.5 Validation of the Final Method

After the method had been established successfully and proven suitable for biopharmaceutical applications the method was validated. Linearity was determined by calibration with external standards in a concentration range of 5-20 µM with a regression

factor of 0.9982. A LOD of 0.6 µM was determined based on a S/N ratio of 3. The migration time precision was good with a RSD of 2.6% and appropriate resolution (R) of G1F/G1F' (R= 1.2) for the assignment of CE-MS signals to CE-LIF. However, the method is not robust with respect to pressure and temperature influences, thus the precise adjustment and control of influencing parameters like capillary inlet height, nebulizer gas flow, ambient temperature and the condition of the ESI needle is required.

3.3 Glycan Identification

ESI-TOF-MS is well suited to characterize charged glycan species. High mass resolution and a fast detection enable the characterization of unknown N-glycans based on their accurate masses by using CE-MS. However, the technique is not capable to distinguish between isobaric substances as galactose and glucose. Thus, compositional information is limited to monomer groups like hexoses, N-acetyhexosamines or desoxyhexoses. In addition no structural information can be obtained firsthand.

For the identification of target glycan structures exact masses using the possible charge states (doubly, triply or four-fold charged) were calculated and used to gain extracted ion electropherograms with a small mass deviation (±5 mDa). The calibrated TOF instrument enabled the determination of accurate masses with a mass deviation of about 5 mDa (about 5 ppm for glycan structures). The MS software (Data Analysis 4.0, Bruker Daltronik) created a simulated mass spectrum for the expected glycan. In cases of uncertainty the isotope distributions were confirmed by overlaying both spectra. The high resolution and accuracy of the TOF instrument is shown by the overlay of a triply charged, APTS-labeled bi-antennary complex-type N-glycan in Fig. 8. For $[M-3H]^{3-}$ the monoisotopic mass of 897.9120 m/z was calculated. The MS determination of the triply charged glycan resulted in a monoisotopic mass of 897.9196 m/z with a mass deviation of 5.8 mDa to the theoretical mass. If necessary the mass spectra can be recalibrated using internal mass calibration, e.g. based on present sodium-EACA clusters or previously known glycans.

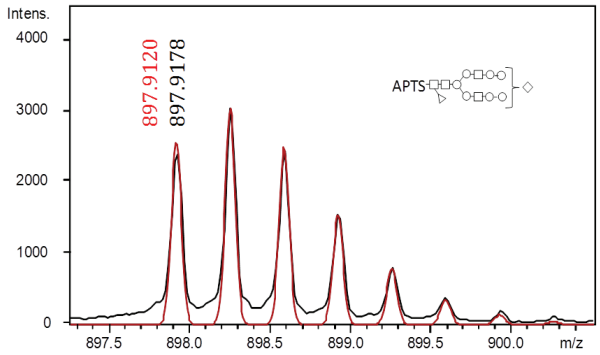

*Fig. 8: Overlay of the measured mass spectrum by MS (**black**) and the calculated mass spectrum (**red**) of a triply charged APTS-labeled glycan*

The analysis of unknown glycan compositions is more challenging. All masses of peaks recognized in the base peak electropherogram are screened for possible glycan masses based on their m/z value and charge state. A software tool was written to calculate all possible glycan compositions for any potential masses of unknown glycans. The boundary conditions of this search were critical, as on one hand possible compositions should be restricted to a reasonable number but on the other hand all possible compositions have to be included. Therefore, the following boundaries have been set for the analysis of N-glycans: mass deviation ±10 mDa, charge state 2-4 and the default glycan monomers referred to the glycan source, based on the core-structure-monomers of two N-glucosamine residues linked to three mannose residues. Thereof, the software creates a list of all possible glycan compositions for the given masses. Thus, in most cases the accurate mass leads to only few potential hits, if the generic glycosylation is known. However, if there is no or few information about the glycosylation mechanism, an prediction solely by accurate mass might be not possible. Especially glycan modifications like sulfation and phosphorylation are difficult to distinguish (mass deviation 9 mDa). Additionally, different monomer combination result in similar or identical mass differences (e.g.: Δm (Hexose + N-Acetylneuraminic Acid) = Δm (Fucose + N-Glucolylneuraminic Acid)).

Furthermore, the accurate mass distances in the charge deconvoluted spectra can help to evaluate the existing glycan monomers, and might give a hint for structural position. The glycan mobility in comparison with known glycans may help to identify the right composition out of the proposal list as discussed in manuscript 3. Thus, all biopharmaceutical samples applied to the CE-MS systems proposed here could be identified. In addition, the identified glycan composition might be confirmed by fragmentation experiments via the cleavage of glycosidic bonds. However, no further structural information like linkage types can be obtained, since for APTS-labeled glycans

hardly any cross-ring fragmentation occurs [91]. Comparison with standards may finally confirm the identification.

3.4 Assignment of CSE-LIF to CE-MS-signals

The assignment of MS to LIF signals is of great importance to identify unknown glycan peaks in LIF, since standards are expensive or even not available. The linear correlation of mobilities of glycans with similar functional groups (e.g. same amount of sialic acids) between different BGE systems, enable the signal assignment and thus, the identification of CSE-LIF signals based on several known glycan species.

A comparison between the proposed acidic and alkaline CE-MS methods and two CSE-LIF methods (using GEL1 and GEL2) was performed with respect to glycan mobilities. APTS-labeled bi-antennary, core-fucosylated complex-type glycans, thereof four non-sialylated and two sialylated glycans, were applied (see Fig. 9).

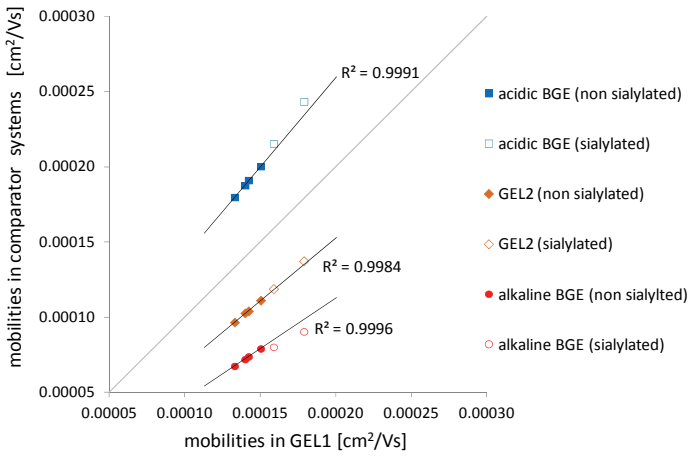

Fig. 9: Glycan mobilities in different BGE systems (acidic and alkaline BGE, GEL2) are plotted against the glycan mobilities in GEL1. ■, ♦, ●: non-sialylated glycans. □, ◊, ○: sialylated glycans.

General mobility shifts between the applied BGE systems were observed due to the different properties. The main impact on mobility differences may have the pH and viscosity. The mobilities of all tested glycans in GEL2 are proportional to the mobilities determined in GEL1. Comparing the alkaline and the acidic BGE to GEL1, the mobilities of the sialylated glycans deviate from the proportionality of non-sialylated glycans.

Thus, the assignment of glycan peaks in the different BGE-systems might be challenging for glycan species of different functionality, since peak shifts and changes in migration order compared to the comparator system are possible. In critical cases (i.e.

numerous unknown signals with similar migration times) several known glycans of similar functionality (like same amount of sialic acids) are required to extrapolate the mobilities and thereby enable the assignment of peaks identified by CE-MS to CE-LIF peaks. This is presented based on the analysis of a recombinant fusion protein (electropherograms are shown in Fig. 3 and paragraph 3.3). The correlation of glycan mobilities in GEL2 and the alkaline BGE was studied (see manuscript 3). The used glycan pool included different glycan structures like complex-type or high mannose-type – sialylated as well as non-sialylated. However, the peaks identified by CE-MS could be clearly assigned to peaks in the CE-LIF approach.

3.5 Detection Sensitivity

The detection sensitivities of the two presented CE-MS systems differ, since the BGE (i.e. high concentration of methanol in the alkaline BGE) and SL compositions (i.e. basic TEA as additive in the acidic BGE) influence ionization efficiency as described in manuscript 3. Thus, higher signal intensities were found for the alkaline CE/MS system (peak height increased by a factor of 3-8).

Additionally, the concentration sensitivities differ strongly between the CE-MS systems and the standard CSE-LIF setup which had been used for the analysis depicted in Fig. 3 performed on a DNA-sequencer (method published by Ruhaak et al. [80]). CE-LIF analysis provides high detection sensitivity due to the selective and highly efficient fluorescence detection for APTS-labeled glycans. The DNA-sequencer adopted for CSE-analysis of APTS-labeled glycans showed a very high sensitivity compared to both CE-MS systems (i.e. 1000-fold more sensitive). For CSE-LIF analysis the CE-MS samples were diluted 1/1000 to adjust the analyte concentrations to the dynamic range of the LIF-detector. An in-line SPE-CE-MS concentration system may enable the analysis of samples previously applied to LIF-detection, as discussed in the next paragraph.

3.6 In-line SPE-CE-MS

The applicable sample concentrations for APTS-labeled glycans using the standard CE-MS systems presented in this thesis are in the low µM-range, to enable the application of lower concentrated samples a novel in-line SPE-CE setup for APTS-labeled glycans was developed based on the acidic CE-MS method.

The in-line SPE-CE setup presents a very effective tool to overcome the drawback of limited concentration sensitivity in CE-MS, as it enables the increase of total injection

volumes from nL to µL. The method is based on a previously established off-line SPE method for sulfated and phosphorylated analytes [92]. The used mix-mode-phase consisting of weak anion exchange and a reverse phase material which had been employed there, was also well suited for the application to APTS-labeled glycans, as initial tests with APTS-labeled glycans on standard cartridges confirmed. The miniaturized in-line SPE construction and the corresponding method were developed based on sulfonic acid standards in an in-line SPE-CE-UV setup. The final setup consists of three capillary segments (outside diameter (od) 365 µm) fritless connected by two polyvinyl-chloride tubings of 190 µm inner diameter (id): i) first segment of 50 µm id and 11 cm length (i.e. distance from inlet to SPE-column), ii) SPE-segment with a larger id of 100 µm and 4 mm length filled with stringed SPE beads (53-90 µm od), iii) separation segment of 50 µm id and 70 cm length.

This construction was used for in-line SPE-CE-MS of APTS-labeled samples employing the acidic CE-MS method (manuscript 4) and PVA-coated capillary segments. Therefore, the SPE column was conditioned with BGE and elution was performed with methanol/ammonia (30 w %) in the ratio of 95/5 (v/v) (detailed procedure see manuscript 4). Finally, an enrichment factor of 800 could be calculated using a 50 nM glycan sample (i.e. peak area equivalent to a 40 µM sample analyzed by CE-MS without SPE). However, for in-line SPE-CE-MS even lower concentrated samples can be applied; the only limiting factor is the required injection time (injection times in the presented work: 5-10 min). Furthermore for APTS-labeled glycans the excess of APTS reagent should be kept as low as possible to avoid reducing bed-capacity (i.e. in this work sufficient linearity was shown up to 2.5 pmol with a 30-fold excess of APTS in solution).

The proposed in-line SPE-CE-MS method enables the identification of APTS-labeled glycans in the sub-nM range, as used in CSE-LIF. Thus, the identification of low abundant glycan species is possible. As future aspect, the system has to be adapted to longer separation capillaries to exploit the high resolving power for APTS-labeled glycans demonstrated here.

Chapter 4

Summary

The aim of this work was the development of novel capillary-electrophoresis/mass spectrometry (CE-MS) methods with high separation power for the characterization of 8-aminopyrene-1,3,6-trisulfonic acid (APTS)-labeled glycans derived from biopharmaceutical products. State of the art for routine glycan analysis is capillary sieving electrophoresis with laser induced fluorescence detection (CSE-LIF) using APTS as a fluorescence label. However, with this technique a firsthand identification of glycans is not possible. Therefore, CE-MS is well suited since it combines the high separation power of CE with the identification capabilities of mass spectrometry due to its selectivity of time-of-flight/mass spectrometry. However, in this field there are no reported CE-MS methods with good reproducibilities and separation efficiencies comparable to CSE-LIF.

This thesis presents two high performance CE-MS methods, with appropriate separation efficiency (i.e. resolution of structural isomers, like G1F/G1F') for biopharmaceutical samples. These include a novel method using an alkaline background electrolyte (BGE) and cathodic detection for the simultaneous analysis of APTS-labeled as well as charged native glycans. The alkaline BGE enables a robust CE-MS application due to the generated electroosmotic flow (EOF), thus minimizing pressure and flow effects at the capillary outlet. The glycans of several different types of biopharmaceutically relevant proteins could be identified. Furthermore, the proposed method enables the determination of derivatization and ionization efficiency.

The second CE-MS method, acidic BGE, was optimized in terms of separation efficiency and reproducibility by investigating potential system affecting parameters (e.g. temperature, pressure changes, capillary positioning and coating, etc.) The developed method is sensitive to pressure and flow effects because the system is not stabilized by an EOF and pressure control at the capillary outlet is missing. Sheath liquid (SL) composition and pressure effects (e.g. nebulizer gas flow and siphoning effects) have the strongest impact on the desired high separation efficiency. For appropriate separation efficiency a basic additive in the SL is mandatory. Out of the tested substances, triethylamine was found to work best. A capillary length of 80-90 cm is required for structural isomer separation, with only minor significance of neutral coatings like polyacrylamide or polyvinyl alcohol coating used in this study. Despite its high sensitivity to pressure and temperature, the resulting method shows good reproducibility (in terms of

migration time stability and separation efficiency), since the main treats as nebulizer gas flow and capillary inlet height are clearly determined. Hence, pressure impacts can be counterbalanced by adjusting these parameters and /or applying the respective pressure difference to the outlet vial.

A systematic study on the signal assignment of glycans, analyzed by two different CSE-LIF methods based on commercial polymers as well as using the proposed CE-MS methods, was performed. Standard CSE-LIF systems apply reverse polarity (anodic detection) like the acidic CE-MS system, in which the overall migration direction is reversed compared to the alkaline system using cathodic detection. The linear mobility correlation of glycans with similar functional groups (e.g. same amount of sialic acids) within the CSE-LIF and CE-MS systems allows the signal assignment and thus the identification of CSE-LIF signals based on several known reference glycans. The signal assignability of different glycan structures (like complex type – high mannose type, sialylated – non-sialylated), identified by the alkaline CE-MS method, to CSE-LIF was demonstrated based on the comparative glycan analysis of a recombinant fusion protein.

The development of a fritless, in-line solid phase extraction (SPE) setup enables the injection of higher sample volumes and concomitant in-line concentration of APTS-labeled glycans. Thus, the limit of detection of the acidic CE-MS method presented here could be decreased from the sub-µM-range to a low nM-range (injection time 5-10 min). The novel bead-string SPE design, lead to an enrichment factor of 800. Prolongation of injection time may further improve the concentration sensitivity. The presented method provides a sufficient linear range (i.e. total sample amount of 2.5 pmol using a 30-fold excess of APTS).

Chapter 5

Zusammenfassung

Ziel dieser Arbeit war die Entwicklung neuer Kapillarelektrophorese/Massenspektrometrie (CE-MS, *capillary electrophoresis/mass spectrometry*) Methoden mit hoher Trennleistung für die Charakterisierung von 8-Aminopyren-1,3,6-trisulfonsäure (APTS)-markierten Glykanen biopharmazeutischer Produkte. Stand der Technik für die Routineanalytik von Glykanen ist die Kapillarsiebelektrophorese mit Laser induzierter Fluoreszenz Detektion (CSE-LIF, *capillary sieving electrophoresis/laser induced fluorescence*) unter Verwendung von APTS als Fluoreszenzmarker. Jedoch ist mit dieser Technik eine direkte Identifizierung der enthaltenen Glykane nicht möglich. Für diesen Zweck eignet sich CE-MS sehr gut, da es die hohe Trennleistung der CE mit der Selektivität der Massenspektrometrie verbindet. Allerdings gibt es in diesem Bereich keine CE-MS Methoden mit guter Reproduzierbarkeit und vergleichbar hoher Trennleistung wie in CSE-LIF.

Diese Thesis präsentiert zwei CE-MS Methoden mit geeigneter Trenneffizienz, (d.h. Trennung von Strukturisomeren wie G1F/G1F') für die Glykananalyse von proteinbasierten biopharmazeutischen Produkten. Hierzu gehört eine neuartige Methode mit basischem Hintergrundelektrolyten (BGE, *background electrolyte*) und kathodischer Detektion für die simultane Analyse APTS-markierter, sowie geladener nativer Glykane. Der basische BGE ermöglicht eine robuste CE-MS Anwendung, da der generierte elektroosmotischem Fluss (EOF) mögliche Druck- und Strömungseinflüsse am Kapillarauslass verringert. Unter Verwendung dieser Methode konnten die Glykane verschiedener biopharmazeutisch relevanter Proteine identifiziert werden. Desweiteren ermöglicht die vorgestellte Methode die Bestimmung der Ionisierungs- und Derivatisierungseffizienz.

Die zweite CE-MS Methode, mit saurem BGE, wurde im Hinblick auf eine hohe Trennleistung und guter Reproduzierbarkeit optimiert, indem mögliche systembeeinflussende Parameter (wie Temperatur, Druck, Kapillarpositionierung und Beschichtungen) untersucht wurden. Das entwickelte saure CE-MS System ist empfindlich gegenüber Druckeinflüssen da das System nicht über einen EOF stabilisiert werden kann und zudem eine Druckregulierung am Kapillarauslass fehlt. Dabei bilden die Zusammensetzung der Schleierflüssigkeit (SL, *sheath liquid*) und Druckeinflüsse (z.B. Vernebelungsgasstrom am Kapillarauslass und mögliche Siphoneffekte) die wichtigsten

Einflussfaktoren auf die gewünschte hohe Trennleistung. Für eine ausreichende Trennleistung sind basische Zusätze im SL von essentieller Bedeutung. Von den getesteten Substanzen war Triethylamin am geeignetsten. Eine Kapillarlänge von 80-90 cm ist nötig, wenn Strukturisomere getrennt werden sollen. Dabei ist die Art der neutralen Kapillarbeschichtung, wie Polyacrylamid oder die hier verwendete Polyvinylalkohol-Beschichtung, von geringer Bedeutung. Die entwickelte Methode zeigt trotz ihrer Druck und Temperaturempfindlichkeit gute Wiederholpräzision (bezogen auf Migrationszeitstabilität und Trennleistung), da die Haupteinflussfaktoren wie Verneblungsgas und Kapillarhöhe am Einlass bekannt sind. Somit können Druckeinflüsse durch Anpassen dieser Parameter und/oder durch Anlegen des entsprechenden Ausgleichsdrucks am Kapillareinlass ausgeglichen werden.

Es wurde eine systematische Untersuchung der Übertragbarkeit der Glykansignale zweier CSE-LIF Methoden (basierend auf zwei kommerziell erhältlichen Polymersystemen) auf die Signale der vorgestellten CE-MS-Methoden durchgeführt. Standard CSE-LIF Systeme verwenden eine anodische Detektion, wie auch die saure CE-MS Methode, wodurch die generelle Migrationsreihenfolge umgekehrt zur basischen Methode mit kathodischer Detektion verläuft. Die lineare Korrelation der Mobilitäten mit ähnlicher Funktionalität (z.B. gleiche Anzahl an Sialinsäuren) in CSE-LIF und CE-MS erlaubt die Signalzuordnung und damit die Identifizierung unbekannter Signale anhand bekannter Glykane. Die Übertragbarkeit der Signale verschiedener Glykanstrukturen (wie “High Mannose” Typ – Komplexer Typ, (nicht) sialysiert – (nicht) fucosiliert), auf CE-LIF wurde anhand der Glykananalyse eines rekombinanten Fusionsproteins mittels basischer CE-MS gezeigt.

Durch die Entwicklung eines fritten-losen, in-line Festphasenextraktions- (SPE, *solid phase extraction*) Systems wurde die Injektion größerer Probenvolumina bei gleichzeitiger Aufkonzentrierung APTS-markierter Glykane ermöglicht. Somit konnte die Nachweisgrenze der sauren CE-MS Methode vom unteren µM-Bereich auf den unteren nM-Bereich (Injektion 5-10 min) herabgesetzt werden. Das neu entwickelte SPE-Bett mit einzeln aufgereihten SPE-Partikeln führte somit zu einem Anreicherungsfaktor von 800. Durch Erhöhung der Injektionszeiten kann die Konzentrationsempfindlichkeit weiter verbessert werden. Die vorgestellte Methode bietet einen ausreichenden linearen Bereich (d.h. Probengesamtmenge bis 2.5 pmol, bei 30-fachem APTS-Überschuss).

Bibliography

1. Walsh, G., *Biopharmaceuticals and biotechnology medicines: an issue of nomenclature.* European Journal of Pharmaceutical Sciences, 2002. **15**(2): p. 135-138.
2. Rader, R.A., *(Re)defining biopharmaceutical.* Nat Biotech, 2008. **26**(7): p. 743-751.
3. Walsh, G., *Post-Translational Modifications in the Context of Therapeutic Proteins: An Introductory Overview*, in *Post-translational Modification of Protein Biopharmaceuticals*. 2009, Wiley-VCH Verlag GmbH & Co. KGaA. p. 1-76.
4. Jefferis, R., *Glycosylation as a strategy to improve antibody-based therapeutics.* Nat Rev Drug Discov, 2009. **8**(3): p. 226-234.
5. Reichert, J.M. and V.E. Valge-Archer, *Development trends for monoclonal antibody cancer therapeutics.* Nat Rev Drug Discov, 2007. **6**(5): p. 349-356.
6. Moutel, S. and F. Perez, *"Antibodies--Europe. Engineering the next generation of antibodies".* Biotechnol J, 2008. **3**(3): p. 298-300.
7. Carter, P.J., *Potent antibody therapeutics by design.* Nat Rev Immunol, 2006. **6**(5): p. 343-357.
8. Apweiler, R., H. Hermjakob, and N. Sharon, *On the frequency of protein glycosylation, as deduced from analysis of the SWISS-PROT database.* Biochimica et Biophysica Acta (BBA) - General Subjects, 1999. **1473**(1): p. 4-8.
9. Kameyama, A., et al., *Strategy for simulation of CID spectra of N-linked oligosaccharides toward glycomics.* Journal of proteome research, 2006. **5**(4): p. 808-814.
10. Jefferis, R., *Recombinant antibody therapeutics: the impact of glycosylation on mechanisms of action.* Trends in Pharmacological Sciences, 2009. **30**(7): p. 356-362.
11. Brooks, S., *Appropriate glycosylation of recombinant proteins for human use.* Molecular Biotechnology, 2004. **28**(3): p. 241-255.
12. Brooks, S.A., *Protein Glycosylation: The Basic Science*, in *Post-translational modification of protein biopharmaceuticals*, G. Walsh, Editor. 2009, Wiley-Blackwell.
13. Lis, H. and N. Sharon, *Protein glycosylation. Structural and functional aspects.* Eur J Biochem, 1993. **218**(1): p. 1-27.
14. Jefferis, R., *Antibody Glycosylation*, in *Post-translational Modification of Protein Biopharmaceuticals*. 2009, Wiley-VCH Verlag GmbH & Co. KGaA. p. 79-108.
15. Hamm, M., Y. Wang, and R. Rustandi, *Characterization of N-Linked Glycosylation in a Monoclonal Antibody Produced in NS0 Cells Using Capillary Electrophoresis with Laser-Induced Fluorescence Detection.* Pharmaceuticals, 2013. **6**(3): p. 393-406.
16. Geyer, H. and R. Geyer, *Strategies for analysis of glycoprotein glycosylation.* Biochimica et Biophysica Acta (BBA) - Proteins & Proteomics, 2006. **1764**(12): p. 1853-1869.
17. Pioch, M., S.-C. Bunz, and C. Neusüß, *Capillary electrophoresis/mass spectrometry relevant to pharmaceutical and biotechnological applications.* Electrophoresis, 2012. **33**(11): p. 1517-1530.
18. Cortes, D.F., et al., *Recent advances in the MS analysis of glycoproteins: Capillary and microfluidic workflows.* Electrophoresis, 2011. **32**(1): p. 14-29.
19. Nakano, M., et al., *Capillary Electrophoresis and Capillary Electrophoresis-Mass Spectrometry for Structural Analysis of N-Glycans Derived from Glycoproteins*, in *Capillary Electrophoresis of Carbohydrates: From Monosaccharides to Complex Plysaccharides*. 2011. p. 205-235.

20. Rakus, J.F. and L.K. Mahal, *New Technologies for Glycomic Analysis: Toward a Systematic Understanding of the Glycome*, in *Annual Review of Analytical Chemistry, Vol 4*. 2011. p. 367-392.
21. Geyer, H. and R. Geyer, *Strategies for analysis of glycoprotein glycosylation.* Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics, 2006. **1764**(12): p. 1853-1869.
22. Hirabayashi, J., A. Kuno, and H. Tateno, *Lectin-based structural glycomics: A practical approach to complex glycans.* Electrophoresis, 2011. **32**(10): p. 1118-1128.
23. Higgins, E., *Carbohydrate analysis throughout the development of a protein therapeutic.* Glycoconjugate Journal, 2010. **27**(2): p. 211-225.
24. Pabst, M. and F. Altmann, *Glycan analysis by modern instrumental methods.* Proteomics, 2011. **11**(4): p. 631-643.
25. Yamada, K. and K. Kakehi, *Recent advances in the analysis of carbohydrates for biomedical use.* Journal of Pharmaceutical and Biomedical Analysis, 2011. **55**(4): p. 702-727.
26. O'Neill, R.A., *Enzymatic release of oligosaccharides from glycoproteins for chromatographic and electrophoretic analysis.* Journal of Chromatography A, 1996. **720**(1-2): p. 201-215.
27. Melmer, M., et al., *HILIC analysis of fluorescence-labeled N-glycans from recombinant biopharmaceuticals.* Analytical and Bioanalytical Chemistry, 2010. **398**(2): p. 905-914.
28. Zauner, G., A.M. Deelder, and M. Wuhrer, *Recent advances in hydrophilic interaction liquid chromatography (HILIC) for structural glycomics.* ELECTROPHORESIS, 2011. **32**(24): p. 3456-3466.
29. Behan, J.L. and K.D. Smith, *The analysis of glycosylation: a continued need for high pH anion exchange chromatography.* Biomedical Chromatography, 2011. **25**(1-2): p. 39-46.
30. Grey, C., et al., *Development of a high performance anion exchange chromatography analysis for mapping of oligosaccharides.* Journal of Chromatography B, 2009. **877**(20-21): p. 1827-1832.
31. Bruggink, C., et al., *Analysis of carbohydrates by anion exchange chromatography and mass spectrometry.* Journal of Chromatography A, 2005. **1085**(1): p. 104-109.
32. Guignard, C., et al., *Analysis of carbohydrates in plants by high-performance anion-exchange chromatography coupled with electrospray mass spectrometry.* Journal of Chromatography A, 2005. **1085**(1): p. 137-142.
33. Melmer, M., et al., *Comparison of hydrophilic-interaction, reversed-phase and porous graphitic carbon chromatography for glycan analysis.* Journal of Chromatography A, 2011. **1218**(1): p. 118-123.
34. Lam, M.P.Y., et al., *Online combination of reversed-phase/reversed-phase and porous graphitic carbon liquid chromatography for multicomponent separation of proteomics and glycoproteomics samples.* Electrophoresis, 2011. **32**(21): p. 2930-2940.
35. Kakehi, K. and Y.-k. Matsuno, *Capillary Electrophoresis and Its Microchip Format for the Analysis of Glycosaminoglycans*, in *Capillary Electrophoresis of Carbohydrates: From Monosaccharides to Complex Plysaccharides*. 2011. p. 83-103.
36. Primack, J., G.C. Flynn, and H. Pan, *A high-throughput microchip-based glycan screening assay for antibody cell culture samples.* Electrophoresis, 2011. **32**(10): p. 1129-1132.
37. Suzuki, S. and S. Honda, *Miniaturization in carbohydrate analysis.* Electrophoresis, 2003. **24**(21): p. 3577-3582.
38. Zamfir, A.D., et al., *Chip electrospray mass spectrometry for carbohydrate analysis.* Electrophoresis, 2005. **26**(19): p. 3650-3673.

39. Zhuang, Z., et al., *Microchip electrophoresis of N-glycans on serpentine separation channels with asymmetrically tapered turns.* Electrophoresis, 2011. **32**(2): p. 246-253.
40. Rabanes, H.R., A.M. Guidote, and J.P. Quirino, *Capillary electrophoresis of natural products: Highlights of the last five years (2006–2010).* Electrophoresis, 2012. **33**(1): p. 180-195.
41. Haselberg, R., G.J. de Jong, and G.W. Somsen, *Capillary electrophoresis–mass spectrometry for the analysis of intact proteins 2007–2010.* Electrophoresis, 2011. **32**(1): p. 66-82.
42. Desiderio, C., et al., *Capillary electrophoresis-mass spectrometry: Recent trends in clinical proteomics.* Journal of Pharmaceutical and Biomedical Analysis, 2010. **53**(5): p. 1161-1169.
43. Suntornsuk, L., *Recent advances of capillary electrophoresis in pharmaceutical analysis.* Analytical and Bioanalytical Chemistry, 2010. **398**(1): p. 29-52.
44. Tseng, H.-M., Y. Li, and D.A. Barrett, *Bioanalytical applications of capillary electrophoresis with laser-induced native fluorescence detection.* Bioanalysis, 2010. **2**(9): p. 1641-1653.
45. Riekkola, M.-L., J.Å. Jönsson, and R.M. Smith, *Terminology for analytical capillary electromigration techniques.* Pure and Applied Chemistry, 2004. **76**(2): p. 443-451.
46. Ruhaak, L., et al., *Glycan labeling strategies and their use in identification and quantification.* Analytical and Bioanalytical Chemistry, 2010. **397**(8): p. 3457-3481.
47. Moyer, S.C. and R.J. Cotter, *Atmospheric pressure MALDI.* Anal Chem, 2002. **74**(17): p. 468A-476A.
48. Kaneta, T., T. Ogura, and T. Imasaka, *Analysis of proteins in biological samples by capillary sieving electrophoresis with postcolumn derivatization/laser-induced fluorescence detection.* Electrophoresis, 2011. **32**(9): p. 1061-1067.
49. Suzuki, H., et al., *Analysis of 1-Aminopyrene-3,6,8-trisulfonate-Derivatized Oligosaccharides by Capillary Electrophoresis with Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry.* Analytical Chemistry, 1997. **69**(22): p. 4554-4559.
50. Guttman, A., F.-T.A. Chen, and R.A. Evangelista, *Separation of 1-aminopyrene-3,6,8-trisulfonate-labeled asparagine-linked fetuin glycans by capillary gel electrophoresis.* Electrophoresis, 1996. **17**(2): p. 412-417.
51. Evangelista, R.A., M.-S. Liu, and F.-T.A. Chen, *Characterization of 9-Aminopyrene-1,4,6-trisulfonate Derivatized Sugars by Capillary Electrophoresis with Laser-Induced Fluorescence Detection.* Analytical Chemistry, 1995. **67**(13): p. 2239-2245.
52. Gennaro, L.A., et al., *Capillary electrophoresis/electrospray ion trap mass spectrometry for the analysis of negatively charged derivatized and underivatized glycans.* Rapid Communications in Mass Spectrometry, 2002. **16**(3): p. 192-200.
53. Klockow, A., et al., *Separation of 8-aminonapthalene-1,3,6-trisulfonic acid-labelled neutral and sialylated N-linked complex oligosaccharides by capillary electrophoresis.* Journal of Chromatography A, 1995. **716**(1-2): p. 241-257.
54. Larsson, et al., *On-line capillary electrophoresis with mass spectrometry detection for the analysis of carbohydrates after derivatization with 8-aminonaphthalene-1,3,6-trisulfonic acid.* Journal of Chromatography A, 2001. **934**(1-2): p. 75-85.
55. Kamoda, S., R. Ishikawa, and K. Kakehi, *Capillary electrophoresis with laser-induced fluorescence detection for detailed studies on N-linked oligosaccharide profile of therapeutic recombinant monoclonal antibodies.* Journal of Chromatography A, 2006. **1133**(1-2): p. 332-339.

56. Sato, K., et al., *Separation of 2-aminobenzoic acid derivatized glycosaminoglycans and asparagine-linked glycans by capillary electrophoresis.* Analytical Sciences, 2005. **21**: p. 21-24.
57. Haselberg, R., et al., *Capillary electrophoresis-mass spectrometry using noncovalently coated capillaries for the analysis of biopharmaceuticals.* Analytical and Bioanalytical Chemistry, 2011. **400**(1): p. 295-303.
58. Maxwell, E.J., et al., *A promising capillary electrophoresis–electrospray ionization–mass spectrometry method for carbohydrate analysis.* Electrophoresis, 2011. **32**(16): p. 2161-2166.
59. Mechref, Y., *Analysis of glycans derived from glycoconjugates by capillary electrophoresis-mass spectrometry.* Electrophoresis, 2011. **32**(24): p. 3467-3481.
60. Ongay, S. and C. Neusüβ, *Isoform differentiation of intact AGP from human serum by capillary electrophoresis–mass spectrometry.* Analytical and Bioanalytical Chemistry, 2010. **398**(2): p. 845-855.
61. Szabo, Z., et al., *Improved sample preparation method for glycan analysis of glycoproteins by CE-LIF and CE-MS.* Electrophoresis, 2010. **31**(8): p. 1389-1395.
62. Horvath, J. and V. Dolník, *Polymer wall coatings for capillary electrophoresis.* Electrophoresis, 2001. **22**(4): p. 644-655.
63. Righetti, P.G., et al., *The state of the art of dynamic coatings.* Electrophoresis, 2001. **22**(4): p. 603-611.
64. Huhn, C., et al., *Relevance and use of capillary coatings in capillary electrophoresis–mass spectrometry.* Analytical and Bioanalytical Chemistry, 2010. **396**(1): p. 297-314.
65. Harvey, D.J., *Analysis of carbohydrates and glycoconjugates by matrix-assisted laser desorption/ionization mass spectrometry: An update for 2007–2008.* Mass Spectrometry Reviews, 2012. **31**(2): p. 183-311.
66. Harvey, D.J., *Analysis of Carbohydrates and Glycoconjugates by Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry: An Update for the Period 2005-2006.* Mass Spectrometry Reviews, 2011. **30**(1): p. 1-100.
67. Lastovicková, M., J. Chmelik, and J. Bobalova, *The combination of simple MALDI matrices for the improvement of intact glycoproteins and glycans analysis.* International Journal of Mass Spectrometry, 2009. **281**(1-2): p. 82-88.
68. Fenn, J.B., et al., *Electrospray ionization–principles and practice.* Mass Spectrometry Reviews, 1990. **9**(1): p. 37-70.
69. Cole, R.B., ed. *Electrospray Ionization Mass Spectrometry: Fundamentals, Instrumentation, and Applications*. 1997, John Wiley & Sons, Inc.: New York.
70. Bruins, A.P., *Mechanistic aspects of electrospray ionization.* Journal of Chromatography A, 1998. **794**: p. 345-357.
71. Gaskell, S.J., *Electrospray: Principles and Practice.* Journal of Mass Spectrometry, 1997. **32**(7): p. 677-688.
72. Smith, R.D., et al., *Capillary zone electrophoresis-mass spectrometry using an electrospray ionization interface.* Analytical Chemistry, 1988. **60**(5): p. 436-441.
73. Kebarle, P. and M. Peschke, *On the mechanisms by which the charged droplets produced by electrospray lead to gas phase ions.* Analytica Chimica Acta, 2000. **406**(1): p. 11-35.
74. Nibbering, N.M.M., *Four decades of joy in mass spectrometry.* Mass Spectrometry Reviews, 2006. **25**(6): p. 962-1017.
75. Amon, S., A.D. Zamfir, and A. Rizzi, *Glycosylation analysis of glycoproteins and proteoglycans using capillary electrophoresis-mass spectrometry strategies.* Electrophoresis, 2008. **29**(12): p. 2485-2507.

76. Campa, C., et al., *Overview on advances in capillary electrophoresis-mass spectrometry of carbohydrates: A tabulated review.* Electrophoresis, 2006. **27**(11): p. 2027-2050.
77. Mechref, Y. and M.V. Novotny, *Glycomic analysis by capillary electrophoresis-mass spectrometry.* Mass Spectrometry Reviews, 2009. **28**(2): p. 207-222.
78. Nagels, B., et al., *Improved sample preparation for CE-LIF analysis of plant N-glycans.* Electrophoresis, 2011. **32**(24): p. 3482-3490.
79. Frisch, E., et al., *Profiling of Endo H-released serum N-glycans using CE-LIF and MALDI-TOF-MS – Application to rheumatoid arthritis.* Electrophoresis, 2011. **32**(24): p. 3510-3515.
80. Ruhaak, L.R., et al., *Optimized Workflow for Preparation of APTS-Labeled N-Glycans Allowing High-Throughput Analysis of Human Plasma Glycomes using 48-Channel Multiplexed CGE-LIF.* Journal of Proteome Research, 2010. **9**(12): p. 6655-6664.
81. Schwarzer, J., E. Rapp, and U. Reichl, *N-glycan analysis by CGE-LIF: Profiling influenza A virus hemagglutinin N-glycosylation during vaccine production.* Electrophoresis, 2008. **29**(20): p. 4203-4214.
82. Gennaro, L.A. and O. Salas-Solano, *On-Line CE-LIF-MS Technology for the Direct Characterization of N-Linked Glycans from Therapeutic Antibodies.* Analytical Chemistry, 2008. **80**(10): p. 3838-3845.
83. Albrecht, S., et al., *CE-LIF-MSn profiling of oligosaccharides in human milk and feces of breast-fed babies.* Electrophoresis, 2010. **31**(7): p. 1264-1273.
84. Jayo, R.G., J. Li, and D.D.Y. Chen, *Capillary Electrophoresis Mass Spectrometry for the Characterization of O-Acetylated N-Glycans from Fish Serum.* Analytical Chemistry, 2012. **84**(20): p. 8756-8762.
85. Liu, Y., O. Salas-Solano, and L.A. Gennaro, *Investigation of Sample Preparation Artifacts Formed during the Enzymatic Release of N-Linked Glycans prior to Analysis by Capillary Electrophoresis.* Analytical Chemistry, 2009. **81**(16): p. 6823-6829.
86. Balaguer, E., et al., *Glycoform characterization of erythropoietin combining glycan and intact protein analysis by capillary electrophoresis - electrospray - time-of-flight mass spectrometry.* Electrophoresis, 2006. **27**(13): p. 2638-2650.
87. Foret, F., et al., *Indirect photometric detection in capillary zone electrophoresis.* Journal of Chromatography A, 1989. **470**(2): p. 299-308.
88. Foret, F., et al., *Liquid sheath effects on the separation of proteins in capillary electrophoresis/electrospray mass spectrometry.* Analytical Chemistry, 1994. **66**: p. 4450-4458.
89. Huhn, C., et al., *Alignment of laser-induced fluorescence and mass spectrometric detection traces using electrophoretic mobility scaling in CE-LIF-MS of labeled N-glycans.* Electrophoresis, 2012. **33**(4): p. 563-566.
90. Belder, D., et al., *Cross-linked poly(vinyl alcohol) as permanent hydrophilic column coating for capillary electrophoresis.* Electrophoresis, 2001. **22**(17): p. 3813-3818.
91. Sandra, K., et al., *Characterization of Cellobiohydrolase I N-Glycans and Differentiation of Their Phosphorylated Isomers by Capillary Electrophoresis-Q-Trap Mass Spectrometry.* Analytical Chemistry, 2004. **76**(19): p. 5878-5886.
92. Bunz, S.-C., W. Weinmann, and C. Neusüß, *The selective determination of sulfates, sulfonates and phosphates in urine by CE-MS.* Electrophoresis, 2010. **31**(7): p. 1274-1281.

Abbreviations

APTS	8-Aminopyrene-1, 3, 6,-trisulfonic acid
BGE	Background electrolyte
BPE	Basepeak electropherogram
CE	Capillary electrophoresis
CFG	Consortium for Functional Glycomics
CSE	Capillary sieving electrophoresis
CZE	Capillary zone electrophoresis
DTT	Dithiothreitol
EACA	ε-Aminocaproic acid
EOF	Electroosmotic flow
ESI	Electrospray ionization
GEL1	Glycan gel separation buffer (Beckman Coulter)
GEL2	POP-7 gel (Applied Biosystems)
HPAEC	High-pH anion exchange chromatography
HPLC	High performance liquid chromatography
IEF	Isoelectric focusing
id	Inner diameter
LIF	Laser induced fluorescence
LN	Low normal
LOD	Limit of detection
LOQ	Limit of quantitation
MALDI	Matrix assisted laser dissociation/ionization
MS	Mass spectrometry
MS/MS	Dual stage ion fragmentation in tandem mass spectrometry
m/z	Mass-to-charge ratio
od	Outside diameter
PAA	Polyacrylamide
PTM	Post translational modification
PVA	Polyvinyl alcohol
QTOF	Quadrupole time-of-flight mass spectrometer
R	Resolution
RSD	Relative standard deviation
SDS	Sodium dodecyl sulfate
SL	Sheath liquid
S/N	Signal to noise ratio
SPE	Solid phase extraction
TEA	Triethylamine
TOF	Time-of-flight mass spectrometer

List of Figures

Declaration of Honour

I herewith declare that I am familiar with the relevant course of examination for doctoral candidates of the Faculty of Biology and Pharmacy of the Friedrich Schiller University Jena.

Further I declare that I composed the thesis myself and that, to the best of my knowledge and belief, the thesis contains no material previously published, except where due reference is made in the text of the thesis. All additional assistance, personal communications, and sources are acknowledged within the work.

I also declare that no other persons except those mentioned have assisted me with the choice and assessment of materials and/or supported me in writing the manuscript. In particular, I did not enlist the assistance of a doctoral consultant and no third parties received either direct or indirect monetary benefits from me for work connected to the submitted thesis.

I declare that I have not already submitted the dissertation as an examination paper for a state or other scientific examination. I also have not submitted the same, a substantially similar, or a different paper as dissertation to another postsecondary school.

Calw, 04. 02. 2014 _____

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Curriculum Vitae

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03/2007 - 08/2007	1st internship: Institute of Forensic Medicine, Uniklinik Freiburg
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List of Scientific Publications

2013 *In-line SPE-CE-MS using a fritless bead-string design – Application for the analysis of organic sulfonates including APTS-labeled glycans*, K. Jooß, J.Sommer, S.-C. Bunz, C. Neusüß, G. K. E. Scriba, Electrophoresis, 2013 DOI: 10.1002/elps.201300388

2013 *Capillary electrophoresis/mass spectrometry of APTS-labeled glycans for the identification of unknown species in capillary electrophoresis/laser induced fluorescence systems,* S.-C. Bunz, E. Rapp, C. Neusüß, Analytical Chemistry, 2013, DOI: 10.1021/ac401930j

2013 *Analysis of native and APTS-labeled N-glycans via capillary electrophoresis-mass spectrometry in the pharmaceutical field,* S.-C. Bunz, F. Cutillo and C. Neusüß in Analytical and Bioanalytical Chemistry, 405 (25): 8277-8284 (2013)

2013 *Isomerization and Epimerization of the Aspartyl Tetrapeptide Ala-Phe-Asp-GlyOH at pH 10 – a Capillary Electrophoresis Study*, C. Brückner, D. Imhof, S.-C. Bunz, C. Neusüss, G. K. E. Scriba; Electrophoresis, 34(18): 2666-2673 (2013)

2013 *The selective determination of sulfates, sulfonates, and phosphates in urine by capillary electrophoresis/mass spectrometry*, S.-C. Bunz and C. Neusüß in Methods Mol Biol 919 :25-34 (2013)

2012 *CE-MS Identification of Amino Acid Sequence Inversion as a New Degradation Pathway of an Aspartyl Model Tripeptide*, C. Brückner, S.-C. Bunz, C. Neusüß, G. Scriba in Chromatographia, 75 (19-20):1205-1210 (2012)

2012 Capillary electrophoresis/mass spectrometry relevant to pharmaceutical and biotechnological applications, M. Pioch, S.-C. Bunz and C. Neusüß in Electrophoresis 33(11):1517-30 (2012)

2010 *CE-MS characterization of negatively charged alpha-, beta- and gamma-CD derivatives and their application to the separation of dipeptide and tripeptide enantiomers by CE*, B. Sungthong, R. Iványi, S.-C. Bunz, G. Scriba in Electrophoresis, 31(9):1498-1505 (2010)

2010 *The selective determination of sulfates, sulfonates and phosphates in urine by CE-MS*, S.-C. Bunz, W. Weinmann, C. Neusüß in Electrophoresis 31(7):1274-1281 (2010)

List of Presentations

Oral Presentation

03/2013	Speed lecture: "Analysis of N-Glycans by Capillary Electrophoresis - Mass Spectrometry" 7th Glycan Forum, Berlin
01/2013	"Analyse von Glykanen mittels Kapillarelektrophorese-Massenspektrometrie im biopharmazeutischer Bereich" 23. Doktoranden Forum, Hohenroda
03/2010	"The selective determination of sulfates, sulfonates and phosphates in urine by capillary electrophoresis-mass spectrometry" 25th International Symposium on Microscale Bioseapartions, Prague

Poster Presentation

03/2013	Analysis of N-Glycans by Capillary Electrophoresis/Mass Spectrometry, S.-C. Bunz, C. Neusüß, 7. Glycan Forum, Berlin
09/2012	In-line SPE-CE for the determination of sulfonic acids, J. Sommer, S.-C. Bunz, C. Neusüß, CE-Forum, Aalen
09/2012	Analysis of Glucosinolates in Arabidopsis Thaliana by Capillary Zone Electrophoresis Coupled to ESI-Mass Spectrometry, T. Balder, C. Steinert, S.-C. Bunz, G. Bringmann, C. Neusüß, CE-Forum, Aalen
09/2012	Identification of the degradation products of Ala-Phe-Asp-GlyOH by CE-ESI-MS C. Brückner, S.-C. Bunz, C. Neusüß, G. K. E. Scriba, CE-Forum, Aalen
09/2010	Capillary Electrophoretic Determination and Characterization of Negatively Charged Cyclodextrin Derivatives Using Mass Spectrometric Detection and Application for Peptides Enantioseparation by CE B. Sungthong, R. Iványi, S.-C. Bunz, C. Neusüß, G. K. E. Scriba, 16th International Symposium on Separation Science, Rome