Native Capillary Electrophoresis-Mass Spectrometry for Protein analysis: Current Progress and Perspectives

Ann-Katrin Schwenzer[1], Lena Kruse[1], Kevin Jooß[2], and Christian Neusüß[1]

[1]Aalen University of Applied Sciences
[2]Northwestern University

March 13, 2023

Abstract

Native mass spectrometry is a rapidly emerging technique for fast and sensitive structural analysis of protein constructs, maintaining the protein higher order structure. The coupling with electromigrative separation techniques under native conditions enables the characterization of proteoforms and highly complex protein mixtures. In this review, we present an overview of current native CE-MS technology. First, the status of native separation conditions is described for capillary zone electrophoresis (CZE), affinity capillary electrophoresis (ACE), and capillary isoelectric focusing (CIEF), as well as their chip-based formats, including essential parameters such as electrolyte composition and capillary coatings. Further, conditions required for native ESI-MS of (large) protein constructs, including instrumental parameters of QTOF and Orbitrap systems, as well as requirements for native CE-MS interfacing are presented. On this basis, methods and applications of the different modes of native CE-MS are summarized and discussed in the context of biological, medical, and biopharmaceutical questions. Finally, key achievements are highlighted and concluded, while remaining challenges are pointed out.

Native Capillary Electrophoresis-Mass Spectrometry for Protein analysis: Current Progress and Perspectives

Ann-Katrin Schwenzer[1], Lena Kruse[1], Kevin Jooß[2], Christian Neusüß[1]

[1] Department of Chemistry, Aalen University, Beethovenstraße 1, 73430 Aalen, Germany

[2] Department of Chemistry and Molecular Biosciences, the Chemistry of Life Processes Institute, and the Proteomics Center of Excellence, Northwestern University, Evanston, Illinois 60208, United States

Abbreviations: ACE, affinity capillary electrophoresis; ACD, antibody-drug conjugate; AmAc, ammonium acetate; AmBicarb ammonium bicarbonate; AmF ammonium formate; BGE background electrolyte; *E. coli, Escherichia coli* ; EPO, erythropoietin; ES, electrospray; FcRn; neonatal Fc receptor; HRN, high resolution native; HSN, high speed native; HOS higher order structure; iCIEF, imaged CIEF; IM-MS, ion mobility-mass spectrometry; IFN-γ, interferon-gamma; IPA, isopropanol; K_d, affinity constant; LIF, light-induced-fluorescence; LPA, linear polyacrylamide; MCE, microchip electrophoresis; MoCE, mobility capillary electrophoresis; msACE mobility shift affinity capillary electrophoresis, nanoSL, nanoflow sheath liquid, nMS native mass spectrometry, nCIEF, native capillary isoelectric focusing; nCZE, native capillary zone electrophoresis; nMCE, native microchip electrophoresis; PB-DS-PB, polybrene-dextran sulfate-polybrene; PVA, polyvinyl-alcohol; SL, sheath liquid; SMIL, successive multiple ionic polymer layer; TD, top-down; TDMS top-down mass spectrometry; UV, ultraviolet.

Keywords:

native capillary electrophoresis-mass spectrometry, biopharmaceutical proteins, protein complexes and conformations, higher order structure, microfluidics

Total number of words : 15185

Abstract

Native mass spectrometry is a rapidly emerging technique for fast and sensitive structural analysis of protein constructs, maintaining the protein higher order structure. The coupling with electromigrative separation techniques under native conditions enables the characterization of proteoforms and highly complex protein mixtures. In this review, we present an overview of current native CE-MS technology. First, the status of native separation conditions is described for capillary zone electrophoresis (CZE), affinity capillary electrophoresis (ACE), and capillary isoelectric focusing (CIEF), as well as their chip-based formats, including essential parameters such as electrolyte composition and capillary coatings. Further, conditions required for native ESI-MS of (large) protein constructs, including instrumental parameters of QTOF and Orbitrap systems, as well as requirements for native CE-MS interfacing are presented. On this basis, methods and applications of the different modes of native CE-MS are summarized and discussed in the context of biological, medical, and biopharmaceutical questions. Finally, key achievements are highlighted and concluded, while remaining challenges are pointed out.

Introduction

Variability in a protein's function arising from a single gene is driven by genetic variation, alternative splicing of RNA transcripts, and PTMs, forming distinct molecular entities, commonly designated as "proteoforms" . In addition, biological functions of proteins are highly associated with their tertiary structures, including protein folding and conformation. Furthermore, proteins regularly do not operate alone but form homomeric (proteoforms of the same base polypeptide sequence) or heteromeric (different protein subunits) protein complexes, dictating quaternary structures. The nature of proteoform structure as well as the composition of a protein complex thoroughly impact functionality and regulation of these biological constructs. A large number of human diseases result for example from structural protein misfolding, protein aggregation, and dysregulations in phosphorylation or glycosylation states, such as Alzheimer disease and Parkinson disease, type 2 diabetes, and cancer . Apart from endogenous proteins, therapeutic, in most cases recombinant, proteins also play a central role in human health as their safety and efficacy strongly relies on constitution and conformation of recombinant protein products. For instance, aggregate formation or protein degradation can drastically reduce the efficacy or may increase immunogenicity of a drug.

Consequently, the characterization of proteins, protein complexes, and associated interactions is indispensable to not only better understand protein function and biological processes at the molecular level but also to better evaluate biotherapeutic protein products. Native mass spectrometry (nMS) is gaining more and more traction as an asset for proteoform and protein complex analysis close to their functional conformational state. Native MS generally enables the analysis of protein complexes by preserving non-covalent interactions with ligands, metals, substrates, and drugs . In comparison with more traditional techniques applied in structural biology, such as NMR spectroscopy, X-ray crystallography, or electron microscopy, nMS does not provide direct molecular structural information . However, it has some major advantages such as speed, selectivity, and high sensitivity, thus, requiring lower amounts of material. Additionally, nMS enables the analysis of complex protein mixtures, especially if coupled to upfront separation techniques. Native MS is typically conducted using solutions close-to-neutral pH to preserve non-covalent interactions . Comparing mass spectra between proteins in their native state to traditional denaturing conditions, several differences can be observed . Due to the preservation of the native protein structure, distinct parts of the polypeptide sequence are not solvent accessible, limiting the number of protonation events resulting in lower and fewer charge states. The concentration onto fewer number of different signals can result in increased sensitivity, particularly for larger proteins. In addition, the higher m/z range accessible by native protein distributions, due to lower charge states, results in higher peak-to-peak resolution which facilitates the detection of smaller mass differences between proteins with similar mass or structure. Nevertheless, nMS is still a technique requiring specific experimental expertise as well as dedicated mass spectrometers for efficient ion transfer and ion separation at the elevated m/z -values .

Unfortunately, ion suppression and overlapping MS signals are often observed in a direct infusion nMS experiment, especially considering complex protein mixtures . In such cases, separation prior to MS analysis can be tremendously helpful to reduce sample complexity. Furthermore, native top-down (TD) protein analysis is becoming more and more popular as it bridges the gap between proteomics and structural biology for in-depth proteoform and protein complex characterization . Stoichiometry and precise subunit composition can be quickly determined and PTMs can be located and characterized using top-down MS (TDMS) under native conditions . In addition, the time-consuming protein digestion step required for bottom-up methods is eliminated. Due to the complexity of proteome samples, high-resolution native separations of samples are indispensable in TDMS for better proteome coverage . Several techniques for separation of proteins under native conditions have been described in the literature . Chromatographic separation approaches compatible with native conditions include SEC , ion-exchange chromatography , hydrophobic interaction chromatography , and affinity chromatography . Electromigrative techniques such as capillary zone electrophoresis (CZE) , microchip electrophoresis (MCE) , mobility capillary electrophoresis (MoCE) , affinity capillary electrophoresis (ACE) , and capillary isoelectric focusing (CIEF) are also well suited for native protein separation. Capillary electrophoresis (CE) exhibits high separation efficiency and resolution and allows due to the low sample consumption the detection of trace levels of analyte . In principle, all these techniques can be performed under native conditions preserving the protein natural conformation. Stand-alone native separations are limited in terms of compositional or proteoforms information. The hyphenation of native separation techniques with MS is beneficial for obtaining information on *e.g.,* protein identity, conformation, metal factors, or stoichiometry also for protein mixtures which has been well summarized in recent reviews . The use of electromigrative techniques to separate analytes under native conditions is well established since the development of blue native PAGE in 1991 . Later, capillary-based techniques like CZE, ACE, and CIEF were used to separate proteins in electrolyte solutions which preserve their higher order structure and allow online native MS coupling.

In this review, we summarize and discuss requirements and opportunities of various native electromigration techniques coupled to native mass spectrometry both in the context of methodologies and applications. In many studies, the native state of proteins and/or proteoforms might have been preserved during separation, even though, this has not been called "native" in most cases (and cannot be verified as such) due to denaturing conditions in the ESI-MS used in the majority of all CE-MS measurements of proteins. Here, we focus on studies where a native state of the protein is preserved also in mass spectrometry. For simplification, we use the term "native" even though "non-denaturing" or "native-like" conditions might be more appropriate. In the first part, adaptations of electromigrative methods to preserve the higher order structure (HOS) of proteins are described covering CZE, ACE, and CIEF separations and their respective microchip variants. Major parameters, such as type and concentration of background electrolyte (BGE), pH, or capillary coating are discussed. After presentation of native ESI-MS conditions, interfacing of electromigrative methods in the context of native protein analysis is discussed. In the following part, methods and applications of CZE-MS, ACE-MS, and CIEF-MS are summarized, finalized by conclusions and perspectives of these emerging techniques. The general concept for these methods is illustrated in Figure 1.

Native conditions in capillary- and microchip-based electromigrative separation techniques

Capillary zone electrophoresis (CZE)

The most common mode of separation in capillary electrophoresis is capillary zone electrophoresis (CZE). After applying an electric field, analytes are separated in fused silica capillaries filled with a conductive solution (the BGE), according to their electrophoretic mobilities. The electrophoretic mobility is a unique characteristic of an ion in a defined system and is proportional to the ion's charge while being inverse proportional to its hydrodynamic radius. Contrary to a denatured protein, in a native, folded state, both the effective surface charge and the hydrodynamic radius of a protein heavily depend on the HOS of a protein . Therefore, determining the effective charge of analytes under certain conditions in order to selectively adjust separation conditions and increase separation performance becomes even more challenging.

In CZE, BGE composition and the type of salt used impact separation performance. Therefore, BGE

components and concentration need to be adjusted in order to obtain the required selectivity and best separation efficiency. In the context of native separation, the BGE also needs to maintain an analyte's HOS in solution. Therefore, BGE composition and pH need to be adjusted in approximation to the preferred physiological environment of the protein of interest . This can also include the addition of specific salts which regulate some protein-protein, protein-ligand, or protein-cofactor interactions . To maintain a native conformation under near physiological conditions, aqueous BGEs like ammonium acetate (AmAc) or formate (AmF), ammonium bicarbonate (AmBicarb), Tris buffer, but also inorganic phosphate and borate buffers are frequently used . Ideally, like BGE components and pH, the ionic strength should mimic the natural protein environment as close as possible. However, physiological ionic conditions are considered to be within 100-200 mM which would lead to high electric currents in a CE system. High currents produce Joule heat inside the capillary causing analyte zone dispersion which reduces separation efficiency. Beyond considerations regarding resolution, excessive heat generation could potentially induce conformational changes or unfolding and therefore needs to be avoided . Separation temperature in general must be considered carefully especially when studying protein interactions as complex stability and integrity are temperature dependent. Typically, the separation capillary is cooled using air or liquids. Additionally, in contrast to optical detectors (*e.g.* , ultraviolet (UV) or light-induced-fluorescence (LIF)), online hyphenation to mass spectrometers is often further limited regarding the current, due to the risk of potential electrical arcing, depending on the interface (see section 3.2). Nevertheless, while offering increased sensitivity mass spectrometry can provide proteoform resolved assessment and charge variant characterization. ESI-MS coupling requires volatile salts such as AmAc to avoid interference with analyte ionization during the ESI process. However, potential effects on analyte stability and complex integrity due to exchanging BGEs should be monitored.

The electrophoretic mobility of an analyte reflects on its charge-to-size ratio. Therefore, unless introducing modifications to the analyte CZE cannot be used to determine charge and size independently. In 2019, a liquid-phase analogue of drift tube ion mobility spectrometry termed mobility capillary electrophoresis (MoCE) was proposed to enable ion separation as well as hydrodynamic radius and effective charge measurements in a single experiment . This can be achieved by applying an electric field for separation in a laminar Poiseuille flow to incorporate Tailor dispersion analysis and electrokinetic dispersion theory . Being able to determine protein charge states in solution is a great advantage as accurate hydrodynamic radii calculations rely on it. Furthermore, protein charge states reflect on protein activity and solubility and potentially give insight into protein conformation. For native-like mobility and effective charge state measurements the electrolyte components (*e.g.* , NaCl or AmAc) need to facilitate and stabilize the folded state of the protein. Analyte separation in MoCE follows the principle of CZE. Therefore, the same ionic strength restrictions due to Joule heating apply to capillary-based applications. In addition, avoiding perturbations of the laminar flow due to temperature gradients is an even more critical factor since these effects can compromise the determination of hydrodynamic radii. Adding nMS to MoCE applications can have the unique advantage to gain low-resolution three-dimensional structural information . In a native mass spectrum, the average charge state of globular proteins correlates with its solvent assessable surface area in solution. Therefore, by combining ellipsoid approximations gathered from MoCE in solution with nMS data information regarding the three-dimensional shape and radius of a protein could be obtained.

Affinity capillary electrophoresis (ACE)

Since its first introduction, the number and versatility of ACE-based methods continuously grow and, along with it, the variety of applications . Affinity studies rely on native interactions between binding partners and to maintain native conformations often phosphate or Tris buffers are used as BGEs . ACE can be applied for the assessment in the free solution and the immobilized mode. However, immobilization of one binding partner introduces the risk of compromising protein structure, thereby interfering with native interactions. Different modes of free solution ACE can be distinguished : the receptor or ligand is either added in varied concentrations to the BGE (*i.e.* , mobility shift ACE, msACE) or to the sample (*i.e.* , pre-equilibrium and kinetic CE) depending on the underlying binding kinetics. The most extensively used mode is msACE, where the mobility shift which occurs upon receptor and ligand complex formation is used to determine affinity constants (K_d). In pre-equilibrium ACE, an already equilibrated mixture of interacting species is injected

into the capillary, free ligand and complex are separated and both amounts are determined and compared to a standard curve . In kinetic CE species interact under non-equilibrium conditions during separation. Binding affinities are temperature-dependant therefore precise K_d measurements require a well-controlled capillary temperature and low Joule heating, as discussed for CZE previously. Commonly optical (*e.g.* , UV and LIF) or electrochemical detectors are applied in ACE. Considering that in msACE affinity constants between specific binding partners are determined, ACE does not benefit as evidently from MS detection compared to other electromigrative techniques. Nevertheless, due to the inherent heterogenous nature of biomolecules and their complexes, even msACE benefits from native MS detection offering protein variant resolved assessment and determination of binding stoichiometry .

As already discussed for CZE, MS coupling requires the use of volatile salts like AmAc to avoid ion suppression. However, altering the electrolyte may influence binding kinetics and therefore should be monitored . Fortunately, ammonium acetate or bicarbonate, at medium pH do not seem to significantly alter protein-ligand interactions . Signal suppression of the complex cannot entirely be avoided when the ligand is continuously fed to the MS (*i.e.* , for dynamic approaches). However, K_d may still be accurately measured without seriously affecting signal intensity when applying ligand concentration in the micromolar range or lower .

Capillary isoelectric focusing (CIEF)

In CIEF, the capillary is filled with different zwitterionic ampholytes (*i.e.* , carrier ampholytes) to form a pH gradient upon introducing an acidic anolyte and a basic catholyte from opposite sides into the capillary. After the analytes are focused by applying an electric field the sample is mobilized commonly either hydrodynamically or chemically using an acid or base to disrupt the pH gradient. In order to circumvent the mobilization step and the resulting loss in separation efficiency, imaged CIEF (iCIEF) using optical detection along the entire capillary is increasingly used . CIEF applications allow to fill the entire capillary with the sample, typically dissolved in the carrier ampholytes and further additives. This increases sample injection volume compared to CZE. For pI determination, pI markers are included in this mixture as well as additives such as methyl or hydroxypropyl cellulose which decrease sample adsorption to the capillary wall, thereby improving peak shape and separation resolution . These mixtures prepared for CIEF applications are often higher in viscosity compared to BGEs typically applied for CZE. This leads to enhanced separation performance by creating a sieving effect, as well as decreased current and reduced Joule heating. This is beneficial when studying labile proteins and protein complexes under native CIEF (nCIEF) conditions. Fonslow *et al.* further assessed the influence of separation temperature on complex stability and observed an increase in stability for their complex of interest by lowering the nCIEF separation temperature to 15°C . They also raised the question of pH-stability of protein complexes in nCIEF which might be critical regarding the pH of the ampholyte mixture as well as during mobilization. In general, protein stability and the potential risk of precipitation is a major challenge in IEF. To avoid protein precipitation at their pI, solubility enhancers such as urea are typically added to the carrier ampholyte mixture. In this way, clogging, unstable current, and other undesired effects impairing the analysis are reduced or negated . Furthermore, the presence of urea also reduces interactions between proteins and the capillary wall . For native measurements it is even more critical to avoid protein precipitation which consequently results in denaturation of the protein's structure. Unfortunately, urea is a chaotropic salt and in high concentrations induces protein unfolding and denaturation . Moreover, the denaturing effect of urea depends on the size of the proteins, structure protection by covalent bonds (*e.g.* , disulfide bridges) as well as the type of underlying interactions forming their HOS. Along with sucrose as a common alternative to urea there are other less chaotropic additives available for CIEF including glycerol and formamide. Both glycerol and formamide are also better compatible with ESI due to less ion suppression compared to urea . To further decrease ion suppression methyl or hydroxypropyl cellulose additives need to be avoided as well. Since ampholytes are essential for IEF the amount of ampholytes can only be minimized. Coupling native CIEF separation to mass spectrometry allows simultaneous pI determination and heterogeneity assessment. Especially the study of protein interactions benefits from the obtained pI information reflecting on surface electrostatic properties of protein complexes .

Microchip electrophoresis (MCE)

Microfluidic chips have gained interest as electrophoretic separation platforms, referred to as microchip capillary electrophoresis (MCE), due to fast and efficient analysis. The use of narrow flat channels with a large width-to-height ratio in microchip electrophoresis has the benefit of more efficiently supporting heat dissipation, thereby reducing Joule heating . As a result, chip-based applications allow higher salt concentrations and increased electrical field strength. Furthermore, the production process of the chip allows a high degree of flexibility in designing and segmenting the glass or organic polymer surface , enabling the integration of additional workflow steps onto the chip . One of the most prominent commercially available microchip devices coupled via ESI to MS is the ZipChip (908 Devices). The manufacturer offers a variety of chips designed for different applications including the high resolution native (HRN) and high speed native (HSN) chip for native protein separations . Additionally, kits for native applications including a generic native antibody kit containing a sample diluent and the background analyte are available. The native antibody BGE consists of water, isopropyl alcohol, AmAc, acetic acid and DL-histidine at a pH of 5.5 .

Due to their clinical relevance for pathogen identification, personalized medicine, and biomarker detection pre-equilibrium ACE applications (*e.g.* , CE-immunoassays) were translated to microchip setups following the lab-on-a-chip concept. Over the years, different reviews have been published which cover on-chip immunoassays development and innovations . Primarily LIF, chemiluminescence, and various electrochemical detectors are used for these applications due to easier miniaturization and lower limits of detection compared to UV/Vis detection . While microchip-based immunoaffinity CE is the most interesting ACE mode for MS coupling, to our knowledge there have not yet reports been published where chip-based ACE applications were directly hyphenated with MS detection.

With the intent to benefit from the same advantages of microfluidic separations discussed for CZE different approaches to microchip-based CIEF and iCIEF have been developed . However, to our knowledge no reports on chip-based CIEF or iCIEF applications specifically designed for native separations have been published so far. For ESI-MS coupling Intabio (now Sciex) has introduced the "Blast" CIEF (iCIEF) microchip in 2019 . The device has not yet been released but native microchip-based CIEF-MS applications are expected in the future.

Capillary and microchip coatings for native separations

With proteins adsorbing to hydrophilic fused silica surfaces commonly all capillary-based electromigrative protein separations require coating of the silica capillary. Especially large and basic proteins are prone to be adsorbed on the silica surface, which decreases, broadens, or even eliminates analyte signals. Another reason for surface coating is the control of the pH-depended electroosmotic flow (EOF) superimposing analyte migration. Pivotal for the magnitude of EOF generated are acidic silanol groups at the contact surface between BGE and silica material (schematically depicted in Figure 2A). It has been thoroughly discussed in literature that the acidic properties of these silanol-groups are highly inhomogeneous . For the three types of silanol groups present on quartz surfaces it has been simulated that the pK_aranges from 4.5 – 5.6 and 8.4-9.3 to >11.3 . Consequently, for native measurements a strong EOF is generated at a physiological near neutral pH.

Uncharged neutral coating agents suppress or even closely eliminate the EOF (Figure 2B) while cationic agents can reduce but also revert the EOF (Figure 2C). Cationic agents are often applied as successive multiple ionic polymer layer (SMIL) to increase the overall coating stability . It should be mentioned that even though both approaches are applicable to native applications the use of coatings for native separations has not yet been as thoroughly evaluated as for denatured proteins. Generally, users can select between dynamic coating reagents such as hydroxypropyl cellulose (often used in CIEF) added to the BGE or applied as a precoating or static covalently bound coating agents. Dynamic coatings can easily be added to the BGE and are commonly used when optical detection like UV/Vis or LIF is employed. Since dynamic coating leaches into the mass spectrometer causing ion suppression in the ESI process as well as contaminating the instrument, only physically or chemically precoated capillaries or chips can typically be used if MS detection

is involved.

Covalently bound neutral coating agents such as polyvinyl-alcohol (PVA) or linear polyacrylamide (LPA) are mostly applied for MS-coupled native CZE, ACE, and CIEF applications. Coated capillaries or microchips are commercially available but can also be prepared in-house. For MCE a chemical vapor deposition method was developed performing very well for protein separation under native conditions, while also providing stable coatings fully compatible with ESI-MS .

Native conditions for CE-MS

Native ESI-MS

Native separation techniques are coupled to MS using ESI, which enables ionization of proteins in the liquid phase by the application of a high voltage . The electric field leads to the formation of charged droplets. Due to solvent evaporation the droplets undergo coulomb fission once the repulsion between charges overcomes the surface tension. This process finally results in the formation of free gas-phase protein ions. In classical ESI-MS experiments, the protein is sprayed from a solution containing organic solvents, such as ACN and low percentages of acid (positive mode) . The organic solvent ensures a fast evaporation of solvent by reducing the surface tension and the acid or base can promote charge transfer to the protein molecules. The redox processes during ESI can significantly affect the pH of the droplets . In the positive ion mode, oxidation reactions such as 2 H_2O 4 H^+ + 4e- + O_2 cause acidification of the solution.

As a liquid phase ionization process, ESI can preserve non-covalent protein interactions. However, this entails several challenges since organic solvents, especially ACN and MeOH, as well as a high or low pH during the ESI process lead to protein denaturation. In addition, non-volatile salts, often used to stabilize proteins in solution (see section 2.1) should be avoided in ESI . They reduce sensitivity and mass accuracy of the instrument, caused by adduct formation, ion suppression and increase of chemical noise by salt cluster ions . Moreover, non-volatile buffers can lead to the formation of salt deposits in the instrument, which can result in a loss of ion transmission . Adduct formation of salt and analyte splits the signal over multiple species, increasing the spectral complexity and reducing the $[M+nH]^{n+}$ intensity, especially for large proteins . If adduct ions of large protein complexes are not resolved, mass accuracy can be dramatically reduced as well as the ability to identify proteoforms with similar masses. Volatile, aqueous buffer systems with physiological pH like AmAc, AmF or AmBicarb can be applied for preserving the native form of proteins as well as for subsequent ESI of analytes . Ammonium acts as a protonating agent and is removed via NH_3 evaporation, thereby avoiding significant adduct formation . Acetate and formate evaporate as acid . Especially for direct infusion experiments, an extensive buffer-exchange to volatile salt solutions prior to ESI is required for proteins stored in non-volatile buffers . Neutral AmAc solutions are most commonly used for native ESI-MS studies. However, AmAc has buffering properties around pH 4.75 and 9.25 but not at neutral pH, expecting a decrease of the pH down to pH around 4.75 during the ESI process (positive ion mode) . Nevertheless, for many proteins non-covalent interactions are still maintained at pH 4.75 and in unbuffered solutions the pH can decrease from the near neutral range even down to pH 1.4 . AmBicarb or AmF buffers can also be applied for native ESI-MS. AmBicarb provides a better buffering capacity at pH 7, however, it may cause denaturation due to CO_2 outgassing . Hedges *et al.*demonstrated that solutions containing bicarbonate exhibit intense foaming when exposed to higher temperatures during the ESI process, leading to unfolding of proteins at the surface of CO_2bubbles prior to ion formation . Thus, the formation of higher charge states is often observed when using bicarbonate buffer systems, however, also depending on the electrospray (ES) potential . The supercharging effect of bicarbonate can also be beneficial, *e.g.* , for coupling studies about protein structure in native solutions to mass spectrometers with limited *m/z* range or to improve MS/MS performance of native protein ions AmF provides buffering around pH 3.75 in the positive ion mode and is thereby even less suitable for buffering in the neutral pH range compared to AmAc . In addition, there is an increased risk of protein denaturation, probably due to its chaotropic nature . In general, ideal ESI conditions keeping the native state depend on the protein and need to be tested. Some proteins and protein complexes also require specific non-volatile salts and the removal of these salts can significantly impact their stability, making the native analysis of these molecules by ESI-MS challenging .

NanoESI is more sensitive and more tolerant towards nonvolatile salts. In nanoESI, flow rates are typically well below 1000 nl/min using emitter with inner diameters of typically <30µm . Thereby, smaller initial droplets are generated, which undergo less solvent evaporation and Coulomb fission . Thus, there is less enrichment of salt, lowering the salt/protein ratio. This significantly decreases the formation of salt cluster ions and salt-adducted proteins. It has been demonstrated that this desalting effect is even more enhanced when using emitters with tip diameters [?]1 µm, enabling direct nanoESI from solutions containing non-volatile salts, such as NaCl, NaCl and Tris-HCl or KCl and Tris-HCl .

As in CE low flow rates (nl/min) occur, it is, in principal, well suited for nanoESI-MS coupling . The different options for interfacing CE with ESI-MS will be discussed in the following section.

CE-MS interfaces for native ESI

CE can be coupled to MS using a sheath liquid (SL) or sheathless interface . In SL techniques, additional liquid closes the electric circuits of both the CE and the ES at the end of the capillary and stabilizes the ES independent of the magnitude of the CE-flow that depends on the EOF . Conventionally, sheath liquids consist of water-organic solvent mixtures with a small amount of volatile acid or base . Organic solvents decrease the surface tension of the SL and increase its volatility. The flow rate of the SL is usually much higher than the eluent from the CE, leading to unwanted dilution, however, the composition of the SL can be utilized to modify the ES chemistry and increase the ionization efficiency . At least two types of SL interfaces can be distinguished: the coaxial SL interface and the nanoflow SL (nanoSL) interface. The coaxial SL interface is traditionally used for the coupling of CE with MS (commercially available from Agilent Technologies). In this configuration, the CE capillary is placed inside a metal needle and SL is coaxially delivered inside this needle. At the sprayer tip, the CE outflow mixes with the SL. The flow rates of the SL are around 1-10 µl/min, requiring typically an additional nebulizer gas and causing high dilution of the analyte, impairing the sensitivity . In addition, the nebulizer gas might cause a suction effect at the capillary tip, potentially decreasing separation efficiency. These disadvantages can be reduced by using a nanoSL interface, which employs reduced SL flow rates (below 1000 nl/min), omitting a nebulizer gas and resulting in improved sensitivity . NanoSL interfaces are characterized by emitters with small ID which are placed close to the inlet of the MS to enhance the transmission efficiency. Since its introduction by Hsieh *et al.* , the Dovichi group improved the nanoSL design , which has later been commercialized by CMP Scientific. Further nanoSL variations have been designed by different laboratory groups , aiming to enhance sensitivity, reproducibility and robustness as well as an easy handling, such as the recently introduced nanoCEasy interface . Unlike the SL interface, sheathless interfaces do not require additional liquid and only use the BGE for spray generation . The most widespread concept is based on a porous capillary tip allowing for electrical contact . The main benefit of this nanoESI technique comprises its sensitivity. MCE can be coupled online to MS using conductive emitter coatings or ion-permeable membranes (analogous to the sheathless method for CE-ESI-MS) or make-up-flow channels (analogous to the SL approach for CE-ESI-MS) . The latter is used in the ZipChip (908 Devices) and the Blast (Intabio/Sciex), respectively.

To transfer proteins and protein complexes in its native structure from the liquid to gas phase by ESI the above-mentioned interfaces can be applied when certain aspects are considered: Organic solvents, such as ACN and MeOH used in SL interfaces often cause protein denaturation . However, even the often used 1:1 mixture of isopropanol and water may keep proteins in their native state, as shown for the antibody infliximab and the glycoprotein antithrombin . Also, the addition of 0.1% acetic acid to the SL can further increase the signal intensity without denaturing proteins such as mAbs . In general, a reduced flow of SL is beneficial to preserve native features of proteins and for minimizing the dilution of the samples . Higher flow rates might favour protein denaturation depending on the composition of the SL, whereas low flow rates cause ES instabilities . The optimal flow rate of the SL also depends on the protein. Marie *et al.* obtained the highest signal intensity for antithrombin using 2 µl/min in a coaxial SL interface , while Le-Minh *et al.* selected a flow rate of 10 µl/min as compromise between signal intensity and structure preservation of mAbs . Depending on the EOF, an ionic boundary at the capillary outlet may be formed in SL interfaces due to the migration of ions from the SL into the capillary, potentially promoting protein denaturation .

NanoSL interfaces are well suited for native ESI-MS and allow the application of purely aqueous SL such as aqueous AmAc solutions . Shen and Kou *et al.* used 25 mM NH_4Ac (pH 6.9) as SL for native SEC-CZE-MS/MS analysis of the *Escherichia coli*(*E. coli*) proteome , whereas 10 mM NH_4Ac have been applied for CZE-MS analysis of the SigmaMAb and proteins compromising the 70S ribosome of *E. coli* . The difficulties of protein denaturation and dilution by the SL can be completely bypassed using a sheathless interface. However, spray conditions are dependent on the BGE and ES conditions cannot be improved by additional liquid . In addition, high currents that often result from high concentrations of salts applied in native CE should be avoided as they can damage the porous tip . Furthermore, salt based BGEs can crystalize at the sheathless spray tip, especially at low flow rates, resulting in a reduction of the ES stability. To solve this problem, either the ES voltage should be reduced or the spray tip should be moved further away from the MS entrance with increasing AmAc concentrations .

The sheathless interface has been applied for native CZE-MS of various native proteins and protein complexes such as mAbs, the ribosomal proteome of *E. coli* and endogenous nucleosomes using the commercially available emitters from SCIEX (CESI 800) . Jooß *et al.* have developed a nCZE-top-down-MS method using sheathless emitters (CESI, SCIEX) for the native analysis of a mixture of proteins and protein complexes from 30-800 kDa .

For the hyphenation of native MCE with MS, 908 Devices offers microchips and BGEs for native antibody analysis . An electroosmotic pump delivers to the end of the separation channel a make-up liquid at composition similar to CE-MS SL-interface, forming native protein ions .

Parameters for native MS

The analysis of native proteins and protein complexes by MS often necessitates specific instrument conditions and adaptations. Efficient transfer of the analyte through the ion optics is crucial while maintaining its native structure and inter- and intramolecular interactions. In the past, standard high-resolution MS instruments designed for small molecules have been modified for native analysis of proteins and protein complexes . Throughout the progression of native MS, commercial TOF, FT-ICR, and Orbitrap instruments with extended m/z range have been developed, such as the Q Exactive UHMR Hybrid Quadrupole Orbitrap Mass Spectrometer (Thermo Fisher Scientific) and the 6545XT AdvanceBio LC/Q-ToF MS (Agilent).

One important finding in native mass spectrometry instrumentation has been that it is beneficial to increase the pressure in the first vacuum stage of the device to analyze large protein complexes . A higher pressure in the front end of the instrument is required for ion cooling through collisions with inert gas molecules. When large proteins enter the ion guides at low pressures, their oscillations result in large deviations from the central ion axis, leading to losses of ions. However, collisions with small gas molecules decelerate large ions (*e.g.* , protein complexes) and, in this way, their transmission is improved . An increased pressure in the instruments front end also promotes desolvation of analytes via collisions with gas molecules . A higher pressure in the ion transfer region can be achieved by reducing pumping efficiency, leaking additional gas into the source region, incorporating a sleeve between the ion guide and the pump orifice to restrict the flow, using heavier background gas, *etc.* . Desolvation can also be promoted by heating up the ion source or accelerating the molecules between the nozzle and the skimmer . Efficient desolvation is necessary to achieve high mass accuracy and to diminish heterogeneity caused by non-specific adduct formation, increasing overall sensitivity . However, excessive pressure (and, therefore, excessive collisions) can lead to a decrease in sensitivity, resulting from complex dissociation and the displacement of ions from the central axis .

The ion optics' electric fields should be finely tuned to maintain the native protein structure and to transfer large constructs that exhibit high m /z values. In this context, radio frequencies of the ion optics are typically reduced to enhance large-ion focusing . In addition, similar to pressure values described in the previous paragraph, excessive acceleration voltage can lead to protein complex dissociation .

In Orbitrap instruments, extended trapping is often required to stabilize larger protein species in the ion routing multipole, however, too much trapping voltage leads to a decline in sensitivity . Fort*et al.* have

demonstrated that in-source trapping can enhance desolvation as well as ion transmission . In this scheme, ions are first trapped in an injection flatapole in the front-end of an Orbitrap instrument. The trapped ions undergo desolvation and lose their initial momentum due to collisions with gas molecules, resulting in improved transmission and mass resolution. Similarly, McGee *et al.* created an in-source-trapping-like scheme on Orbitrap Tribrid instruments that additionally filters low-mass contaminants via a so-called "voltage rollercoaster" . Low-mass contaminants can suppress the spectral quality of protein analytes via space charge effects, and their removal improves the analysis of high $m\ /z$ species.

In the context of Q-TOF instruments, Shen *et al.* investigated the fragmentor and skimmer voltage as well as the collision energy in the CID cell for native analysis of mAb SigmaMAb . They increased the fragmentor and skimmer voltage for enhanced salt and water declustering resulting in improved sensitivity. In general, antibodies tolerate a higher collision energy compared to smaller molecules, however, an increase of the collision energy decreased mAb signal intensities in this case, likely due to analyte fragmentation from excess energy. Similar to the pressure within the MS system, the amount of energy analytes are exposed to must be finely balanced to achieve efficient ion transmission. There are no broadly optimal gas pressures or radio frequencies since these values are very instrument and analyte dependent , but parameter settings reported in previous studies can serve as guidance for an appropriate range of values, especially if similar instrument setups are utilized. Nevertheless, optimal MS parameters can vary significantly between analytes in native mode compared to the more general applicable settings commonly encountered using denaturing conditions.

Methods and applications of native electromigrative techniques coupled to nMS

Methods of nCZE-nMS

The growing number of publications in recent years has demonstrated the efficiency of CZE-MS for the analysis of protein constructs in their native conformations. In addition, a series of CZE separations might have been conducted preserving native features of proteins without being recognized by the user. Potential examples are the analysis of erythropoietin (EPO) or recombinant human growth hormone by CZE-MS. However, these features were not reflected in the resulting mass spectrum due to the applied denaturing conditions during the ESI process . Before 2010, only the coaxial SL interface from Agilent was commercially available and thus, utilized by a majority of laboratories as CE-MS interface. This setup relies on diluting down the original CE flow drastically in a so-called sheath liquid, which typically contains a high amount of organic solvent, resulting in denaturing conditions. In addition, the number of mass spectrometers adapted for the transfer of higher m/z ions was comparably limited. Here, we will focus on methods and applications where both, CE and MS were performed under native conditions.

A general advantage of CZE compared to a more traditional native direct infusion experiment is that non-volatile salts can be separated from proteins using CZE prior to MS analysis, thereby circumventing an extensive buffer exchange of proteins stored in non-volatile buffers . For example, sodium tends to migrate very early in the electropherogram being detected as sodium acetate clusters. In addition, the separation of proteins, protein complexes, and aggregates in native CZE more closely reflects their solution based conformations and aggregates compared to solely relying on direct infusion which can be prone to artefact formation during the ESI process and bias towards certain species based on ion transfer settings .

Publications describing methods and applications of native protein analysis by CZE-MS are summarized in Table 1. Most commonly, BGEs based on aqueous AmAc have been applied, typically containing 10 – 50 mM AmAc at pH 6 – 8. For instance, Le-Minh *et al.* concluded for infliximab that a BGE of 40 mM AmAc and pH 6.0 is the best compromise between signal intensity and preservation of the native mAb conformation . In addition, aqueous AmBicarb-based solutions have been applied by Bertoletti *et al.* for the analysis of $Beta_2$-microglobulin . To optimize separation conditions, different BGE's as well as different salt concentrations in the BGE should be compared and evaluated, as demonstrated in Figure 3 for (A) a mAb and (B) a mixture of four model proteins. Comparing 10 mM AmAc and AmF (Figure 3 A), AmAc showed slightly better separation regarding the peaks labelled with a black box. 50 mM AmAc resulted in

a longer migration time and wider peaks compared to 10 mM AmAc. In Figure 3 B, separation improved with increasing AmAc concentration. However, it should be kept in mind that high salt concentrations can lead to high CE currents which can be detrimental for the interface set up.

In the coaxial SL interface a SL containing water and organic solvent, such as MeOH and IPA has been applied . Le-Minh *et al.* compared the influence of different SLs, ACN, MeOH and IPA mixed with water in a ratio of 50:50 v/v for the mAb infliximab and observed that ACN and MeOH induced denaturation. For IPA, denaturation, and the associated shift in the charge state distribution, was drastically reduced. Sometimes formic, acetic acid or AmAc is further added to the SL. For nanoSL interfaces from CMP, aqueous AmAc solutions were applied . Considering sheathless CE-MS interfaces, mainly the porous tip interface was employed . Regarding capillary coatings, neutral coatings are most commonly applied for nCE-MS to reduce protein adsorption to the inner CE capillary wall and to lower the EOF and thereby, increase the resolution between proteins (see above). Usually LPA and PAM , but also PVA coatings have been utilized . Some publications also mention the successful application of bare-fused silica capillaries for nCZE-MS . In this context, Belov *et al.* compared the performance of bare-fused silica and PAM coated capillaries and concluded, that the PAM coated capillary demonstrated a better reproducibility and higher efficiency for more complex samples . Furthermore, SMIL coatings are a promising alternative to the more traditional single layer coatings. For instance, Le-Minh *et al.* used a cationic polybrene-dextran sulfate-polybrene (PB-DS-PB) coating and applied reversed polarity to create a strong EOF towards the mass spectrometer. However, under these conditions, H^+-ions from the SL can migrate into the separation capillary leading to the formation of an ionic boundary .

In 2021, Jooss *et al.* provided standard operation procedures for the analysis of model proteins and protein complexes ranging from 30 to 800 kDa by nCZE-TDMS for different Orbitrap instruments using a porous tip interface. Various parameters for nCZE-TDMS are discussed in detail, including the influence of BGE compositions, separation voltage, supplemental pressure, and MS parameters on the quality of the spectra as well as optimized fragmentation conditions for native top-down analysis of intact complexes. This protocol can be used as a guide to optimize separation and MS parameters for the analysis of native proteins and protein complexes .

Applications of nCZE-MS

Denaturation, misfolding, dimerization, or aggregation of antibodies can impact the efficacy and safety of therapeutic antibodies and may increase their immunogenicity, making an extensive characterization of therapeutic drugs essential before patient administration. Thus, the characterization of biopharmaceuticals such as therapeutic mAbs is an important field of application for native CZE-MS. Belov *et al.*analyzed a mAb in 2017 and 2018 under native conditions and detected the monomer and also traces of a dimeric species of the respective mAb . However, the dimer could have also been formed during the ESI process as the dimeric species co-migrated with the monomeric form of the mAb. Le Minh *et al.* successfully analyzed the mAb infliximab by nCZE-MS under native conditions and detected simultaneously different conformational states including native and unfolded monomers as well as dimers of the mAb in a storage stressed sample (6 months at 4 degC) . To provide additional evidence that the dimers observed by nCZE-MS are not formed artificially during ESI, they confirmed their experiments by atomic force microscopy. In 2021, Shen *et al.* developed a native CIEF-assisted CZE-MS method for the analysis of mAbs . After injection of the samples, they first applied native CIEF stacking to focus the mAb and increase overall sensitivity. After completion of the focusing step the antibody was further separated under native conditions by CZE. In this way, it was possible to increase the sample loading capacity without loss of separation resolution. They successfully characterized glycoforms, variants, and aggregates of two different mAbs.

Apart from mAbs, the characterization of other proteins and protein complexes plays a pivotal role in biopharmacy, either to characterize the quality of a protein drug or to get a better understanding of biological processes in cells. The plasma glycoprotein antithrombin is used as a drug for patients with hereditary antithrombin deficiency . Only the native form of antithrombin is active and the presence of latent or dimeric forms decreases the quality of therapeutic antithrombin products making a fast and reliable quantification

of different protein forms necessary. Marie *et al.* successfully differentiated between the native, latent, and dimeric forms of antithrombin using CZE-MS.

Besides analyzing single proteins and complexes, whole cellular proteomes can be analyzed by native CZE-MS/MS. Shen *et al.*prefractionated lysed *E. coli* cells using size-exclusion chromatography and then analyzed the collected fractions by CZE-MS/MS, which resulted in the identification of 144 proteins, 672 proteoforms and 23 protein complexes . Though of note is that so far, there is no efficient way to carry out elaborate data base searches for non-targeted native top-down proteomics. In 2020, Mehaffey *et al.*characterized the *E. coli* 70 S ribosome at various Mg^{2+} concentrations by CZE-MS/MS without previous fractionation. The magnesium cation was required to maintain the 3D structure of the ribosome, highlighting the ability to perform native CZE-MS using such additives. 500 µM magnesium acetate resulted in the observation of the intact 30S and 50S subunits while a reduction of the magnesium concentration and the removal of rRNA led to observation of subcomplexes and single proteins of the ribosome (see Figure 4). They identified 48 of the 55 *E. coli* ribosomal proteins as 84 proteoforms including 22 protein-metal complexes and 10 protein-protein complexes .

Jooß *et al.* generated a platform for the separation and characterization of whole nucleosomes as well as their histone subunits and PTMs using nCZE-TDMS . Their platform allowed a direct injection of nucleosome samples in complex buffers into the CZE system without previous sample preparation. Therefore, they developed a "standard" method and a "high-resolution" method by decreasing the supplemental pressure and thereby increasing the run time to maximize the resolution if necessary. This resulted in significantly improved resolution and better separation of the tetra-acetylated (H3K4,9,14,18a) and ubiquitinated (H2BK120ub) Nucleosomes, as shown in Figure 5.

nMCE-nMS

In native MCE-MS applications, the microfluidic CE separation was carried out on a ZipChip platform using the HRN microchip, the BGE provided in the ZipChip Native Antibody kit and the ZipChip interface from 908 Devices (see Table 1). The BGE is a proprietary formulation based on AmAc at pH 5.5, which is modified with 4% DMSO before usage . The microchip channels are modified with a covalent coating to minimize adsorption of proteins and suppress the EOF . Until now, native MCE-MS using the ZipChip platform was only applied for the characterization of mAbs, especially for the analysis of native intact mAb charge variants , as summarized in Table 1. For instance, Carillo *et al.* analyzed the three widely used IgG1 mAbs rituximab, bevacizumab, and trastuzumab and identified 52 proteoforms of trastuzumab as well as fragments and sialylated *N* -glycans of rituximab . The analysis of mAbs charge variants is an essential part of critical quality attributes (CQA) to ensure efficacy and safety of a therapeutic product. Especially for this purpose, the ZipChip MCE-MS platform offers a fast and simple application platform requiring minimal sample preparation and method optimization . The application of this set up to other classes of proteins and protein complexes is promising, but still needs to be explored in the future.

ACE-nMS

All ACE-nMS approaches were based on mobility shift ACE (see Table 1). To adapt ACE separations to MS detection different BGEs were selected in accordance with the pI and stability of the analyte as well as their binding affinity. Sufficient separation and ionization of the respective analytes was observed using BGEs containing 25 – 50 mM AmAc at pH 6-8 . Domínguez-Vega *et al.* applied a triple layer SMIL coating of polybrene-dextran sulfate-polybrene (PB-DS-PB) and used a coaxial SL interface for their measurements [42]. They added cortisone as a neutral EOF marker to determine μ_{eff}. The presence of an EOF made it also necessary to add 25 mM AmAc (pH 8.0) to the isopropyl alcohol-water SL to avoid ion depletion at the end of the capillary. Gstöttner et al. used a porous tip sheathless interface equipped with OptiMS neutrally coated capillaries (Sciex) . Domínguez Vega *et al.* observed slightly higher K_d values in ACE-MS compared to ACE-UV. They attributed this to an increase in temperature due to the nature of the coupling leaving a large part of the capillary without adequate temperature control, required for precise K_d determination. With the exposure of the capillary to the (warm) ion source, the section closest to the MS is especially prone

to temperature increases.

In their initial work , Domínguez-Vega *et al.* developed and evaluated an msACE-nMS method for simultaneous heterogeneity and affinity assessment based on trypsinogen variants and their aprotinin complexes. They separated and singly, doubly, and triply deamidated trypsinogen charge variants at pH 8. Furthermore, MS detection enabled binding stoichiometry assessment as well as the analysis of comigrating compounds. For quantitative assessment of the trypsinogen-aprotinin interaction, K_d values were determined based on the extracted ion electropherograms. Accuracy of the obtained values was evaluated by comparison with ACE-UV measurements using the same BGE. In principle, K_d values can also be obtained based on the abundance of the complex relative to the signal of the free ligand in the mass spectrum offering mass and K_d determination for comigrating substances. However, higher K_d values with increased standard deviation were observed, compared to those based on electrophoretic mobility shift. By comparing the MS-based results to direct infusion experiments they confirmed that the discrepancy can be attributed to the MS detection. The authors concluded that different ionization efficiencies of the free protein and the complex as well as varying complex stabilities between the liquid and the gas phase could be the reason. This observation highlights again the importance of having a native separation system, which can reflect interactions more adequately taking place in solution compared to direct nMS analysis. To demonstrate the use of their method for fast affinity screening of heterogeneous proteins they also analyzed trypsin and α-chymotrypsin in the absence and presence of aprotinin.

In 2021, Gstöttner *et al.* published the first msACE-nMS approach for functional studies on mAbs. Due to their importance for antibody therapeutic pharmacokinetics, they focused on interactions between the neonatal Fc receptor (FcRn) and antibodies as a modal system. In this work, different engineered antibodies and oxidized samples were analyzed. The hyphenation to nMS allowed simultaneous monitoring of antibody as well as FcRn heterogeneity, individual binding assessment and complex stoichiometry determination. The mobility shift of the H_2O_2-stressed and the reference mAb due to the presence of FcRn is illustrated in Figure 6A. The data indicated a decrease of the FcRn affinity with antibody oxidation and a glycosylation effect, with slightly higher affinities for galactosylated glycoform (Figure 6B). No significant effect of the type of FcRn glycoform on complex formation could be observed, suggesting no influence of the FcRn glycosylation on antibody binding. MS investigation on binding stoichiometry revealed 1:1 and 1:2 mAb/FcRn complexes under the applied conditions. The use of differently engineered Fc constructs further enabled the differentiation between symmetric and asymmetric binding. In 2022, Gstöttner *et al.*further expanded their work and published a detailed study on glycoform binding assessment with msACE-nMS to study the binding of different antibody glycoforms to the FcγRIIa receptor . Clear differences in binding between doubly-, hemi-glycosylated, and non-glycosylated antibodies, as well as for mutated IgG1 antibodies silenced for Fcγ binding, were observed. Moreover, they reported for the first time that high mannose glycoforms show a decrease in affinity for FcγRIIa.

nCIEF-nMS

Native CIEF Methods and applications involving nMS are summarized in Table 2 . To limit ion suppression ampholyte concentrations are generally reduced to 1 – 1.5% (v/v). Furthermore, any additives are omitted except for glycerol. Partial filling of the capillary with catholyte/proteins-ampholyte mixtures enables direct focusing and subsequent mobilization by providing electrical continuity throughout the analysis . 50 mM or 0.1% formic acid as a anolyte and 100 mM ammonia or 10 mM AmBicarb as catholyte were used. Small quantities of glutamic acid and arginine were added to detect the pH gradient limits . To enhance analyte solubility up to 40% glycerol were added to the water or AmAc (10 mM) based carrier ampholyte mixture depending on the sample. The high viscosity of the glycerol solution has the additional benefit of an anticonvective effect. This decreases analyte adsorption, and also results in a reduced EOF. The addition of glycerol allowed Mokaddem *et al.* to use even bare fused silica instead of LPA coated capillaries, compared to other research groups. While this could be attributed to the lack of more stable coatings at that time, it substantiates the advantage of glycerol as a solubility enhancer for hydrophobic and basic proteins.

A remaining challenge is mobilizing analytes without sacrificing separation efficiency, for example due to

peak broadening resulting from hydrodynamic mobilization. Therefore, all authors maintained the focusing voltage during pressure mobilization. Stable spray conditions and efficient ionization were achieved using different sheath liquids either composed of 10 mM AmAc (pH 5) , and AmAc (pH 5) with the addition of 10% (v/v) ACN , or by using a mixture of equal amounts of methanol and water and 1% (v/v) acetic acid . Xu *et al.* observed broader distributions and slightly higher charge states compared to nCZE-MS results and attributed this to a supercharging effect caused by glycerol. They also described effective desalting of holomyoglobin with the addition of 15% glycerol resulting in comparably clean mass spectra.

Mokaddem *et al.* developed and evaluated a nCIEF-nMS method for a mixture of standard proteins commonly used as CIEF pI markers . In 2015 Przybylski *et al.* applied nCIEF-nMS for the characterization of the highly basic cytokine human interferon-gamma (IFN-γ) as well as its active non-covalent homodimeric form . Accurate focusing of protein variants with close pI values was achieved as well as pI determination of the homodimer. Furthermore, a strong contribution of the two regulatory C-terminal clusters of basic amino acids (*i.e.* , D1 and D2) to the positive electrostatic potential of the protein dipole was confirmed. Comparing single and deletion mutations within these regions enabled the investigation of the impact of these domains on the pI values which might reflect on different expositions of the domains and the flexibility of the C-terminal domain. They proposed that their results could correlate with the proposed mechanism of the interaction and binding of D1 and D2 to heparan sulfate.

In 2022 Xu *et al.* published a nCIEF-nMS method for microheterogeneity assessment of streptavidin and the carbonic anhydrase-zinc complex, in comparison to nCZE-nMS. The group achieved liquid-phase separation and characterization of seven different forms of a streptavidin homotetramer with different PTMs (i.e., N-terminal methionine removal, acetylation, and formylation) (Figure 7). In addition, various carbonic anhydrase-zinc complex proteoforms could be detected in a separate experiment (succinimide, deamidation, etc.) and characterized regarding their individual pI-values. Moreover, the applied their method was applied to an interchain cysteine-linked antibody-drug conjugate (ADC), partially resolving drug-to antibody ratio species.

Conclusions and Perspectives

Native CE-MS has gained quite some attraction recently. With the different approaches available, efficient separation can be combined with detailed mass spectrometric information for the structural analysis of proteins and protein complexes in biological, medical, and biopharmaceutical context. In contrast to most LC-MS strategies, such as RPLC, CE-MS can be performed under native conditions with comparably minor efforts and allows a more efficient separation with narrower peaks. Furthermore, the general benefits of CE, such as low sample requirement and fast separation, apply and can be advantageous. More importantly, the selectivity of electromigration techniques allows to separate proteoforms or protein complexes with minor differences in charge and/or size (CZE-MS), according to small differences in pI (CIEF-MS), or protein-target-affinity (ACE-MS). ACE-MS even allows to determine binding constants accurately.

With the advent of more efficient CE-MS interfaces as well as more sensitive mass spectrometers over the last two decades, intact protein analysis by CE-MS technology became generally more accessible, which includes the more recent native CE-MS developments. Previously, native separations might have been already performed in many cases, however, not detected as such, due to denaturing ESI-MS conditions (*e.g.* , sheath liquid composition). Additionally, the development of various capillary coatings has been substantially contributing to nCE-MS methods and applications. Moreover, nanoESI interfaces, either using a SL or performed sheathless, played a pivotal role in the progress of nCE-MS, due to increased sensitivity, increased matrix tolerance, and ease-of-use. Furthermore, our understanding of the properties of the "native" ESI process increased regarding mechanism and efficient analysis of large proteins and protein complexes. Still, there is no general setup that works for every protein or protein complex of interest, and major parameters in both the separation and the ESI-MS need to be carefully adjusted according to stability of the HOS of interest.

In this way, new fields of applications have been opened for the various kind of native capillary electro-

migration techniques coupled to native ESI-MS. CZE-MS has been used in several applications to analyze proteoforms of therapeutic proteins, especially mAbs. Besides the success of these targeted approaches, a truly non-targeted native CE-TDMS approach to analyze proteoforms of native constructs in a proteome-wide fashion is of major interest and will be possible when adequate software tools for data generation, database search and analysis are available. This will advance CZE-MS further towards native TD proteomics. Native CIEF-MS has also been developed with a focus on biopharmaceutical targets. This mode of CE separation adds another layer to native protein construct analysis by providing accurate determination of the respective pI-values. ACE is a major topic since decades and is*per se* applied under native conditions. ACE-MS has been shown to give highly important information on proteins as it combines the characterization of proteoforms with its biological and/or therapeutic effect.

In the future, the addition of mobility in the gas phase using modern ion mobility-mass spectrometry (IM-MS) techniques may add valuable information, especially considering the analysis of different conformational states. So far, IM-MS has been used to characterize native proteins and protein complexes , whereas the online coupling of mobility in liquid phase(CE) and in gas phase (ion mobility) was only realized just recently, focusing on carbohydrates and small molecules .

Over the coming years, we expect a significant increase in methods and applications of native CE-MS to tackle the various challenges of in-depth protein and protein complex characterization in the context of various different fields, such as biopharmaceuticals, structural biology, and many more.

Acknowledgments

This study was funded by the German Federal Ministery for Education and Research within the ProCEVen project (FKZ 13FH135KX0).

Conflict of interest

The authors declare no competing financial interest.

Figures

Figure 1 : Characterization of proteins using denaturing CZE conditions and denaturing ESI-MS as well as native CZE, native CIEF and ACE coupled to native MS, respectively.

Figure 2 : Representation of the migration of cations in a CE-MS setup depending on capillary coating, the resulting EOF and for the required polarity. (A) shows the migration in an uncoated, (B) in a neutrally coated capillary and (C) using a cationic (SMIL) capillary coating. The length of the arrows represents the strength of the EOF (green), the magnitude of analyte mobility (orange) and the magnitude of the resulting apparent mobility (blue).

Figure 3. Evaluation of BGE composition on separation performance of native CZE-MS. (A) Base peak electropherograms for SigmaMab with 10 mM AmAc (AA), 10 mM AmF (AF), and 50 mM AmAc as the BGE. The peaks labeled with black boxes in the electropherograms represent the same mAb species. (B) Extracted ion electropherograms (EIEs) of four different model proteins separated using 20, 40, and 60 mM AmAc as BGE, respectively. Separation improved with increasing AmAc concentration. EIEs for the proteins were created using the three highest charge states, respectively. Adapted and reprinted with permission from ref (A) and (B). Copyright © 2021, Elsevier.

Figure 4. (A) Base peak electropherogram of *E. coli*ribosomal proteins with 500 µM magnesium acetate in the BGE. (B) Corresponding mass spectra of the dominant species in (A) collected at (1) 53.72 and (2) 65.41 min allowed these species to be identified as the 30S and 50S subunits. Base peak electropherograms of subcomplexes and single proteins after rRNA removal with (C) no Mg^{2+} and (D) 100 µM Mg^{2+} in the BGE. Reprinted with permission from ref . Copyright © 2020 ACS, American Chemical Society.

Figure 5. EIEs of a sample containing H2BK120ub and H3K4,9,14,18ac analyzed with (a) standard (5 psi supplemental pressure) and (b) high-resolution (2.2 psi supplemental pressure) CZE methods. c-f: corre-

sponding mass spectra generated by averaging the EIE peaks. Reprinted with permission from ref . Copyright (c) 2021, American Chemical Society.

Figure 6. Example for an ACE-nMS application showing a sheathless CE-MS separation obtained for a H_2O_2-stressed (A) NGmAb or (B) AAA-mAb sample using a BGE without FcRn (upper panel) and containing FcRn (lower panel). The blue trace corresponds to the extracted ion electropherograms (EIEs) of the double-oxidized /two red stars), the green trace corresponds to the EIEs of mono-oxidized (one red star), and the brown trace shows the EIEs of the non-oxidized mAbs present in the mixture. Signal intensities are normalized. Reprinted with permission from . Copyright (c) 2021 The Authors. Published by American Chemical Society. Licenced under CC BY-NC-ND 4.0.

URL: https://pubs.acs.org/doi/10.1021/acs.analchem.1c03560.

Figure 7. Example for a nCIEF-nMS application. (A) Base peak electropherogram of streptavidin (SA) analyzed by nCIEF-MS; (B) deconvoluted mass spectra of seven SA charge variants separated from nCIEF-MS. The inserted figure shows the zoomed-in EIEs for the overlapping variants. Adapted and reprinted with permission from . Copyright (c) 2022, American Chemical Society.

Table 1: Analysis of proteins using native CZE-MS, including MCE-MS and ACE-MS.

Separation mode	**Separation conditions**	**Coating**	**Interface**	**Sheath liquid / Make-up liquid**	**MS instrument**	**Analyte**	**Ref.**
CZE	BGE: 50 mM AmAc (pH 8.3); normal polarity (30 kV)	uncoated	Coaxial SL	10 mM AmAc (pH 6.8)/ MeOH (50:50, v/v)	TOF (Mariner)	Myoglobin	
CZE	BGE: 50 mM AmAc (pH 7.4); reverse (20 kV), 0.7 psi	PVA	Coaxial SL	IPA/water (50:50, v/v) containing 14 mM AmAc	QTOF (micrOTOFQ, MAXIS H/D)	Antithrombin	
CZE	BGE: 50 mM AmBicarb (pH 7.4); normal polarity (30 kV)	uncoated	Coaxial SL, sheathless porous tip	IPA/water/formic acid (49.5:49.5:1, v/v/v)	TOF (6210 LC/MS), QTOF (maXis HD Ultra high resolution)	Beta2-microglobulin	
CZE	BGE: 40 mM AmAc (pH 7.5); normal polarity (20 kV), 1-3 psi	PAM	Sheathless porous tip	not applicable	Orbitrap (QE-EMR, QE Plus)	*E. Coli* ribosomal extract, standard proteins, mAb: trastuzumab	

Separation mode	**Separation conditions**	**Coating**	**Interface**	**Sheath liquid / Make-up liquid**	**MS instrument**	**Analyte**	**Ref.**
SEC-CZE	BGE: 50 mM AmAc (pH 6.9); normal polarity (15 kV), 1 psi	LPA	NanoSL	25 mM AmAc (pH 6.9)	Orbitrap (QE HF)	*E. Coli*	
CZE	BGE: 40 mM AmAc (pH 7.5); normal polarity (20 kV), 3 psi	PAM	Sheathless porous tip	not applicable	Orbitrap (QE Plus)	mAb: IgG1	
CZE	BGE: 40 mM AmAc (pH 6.0); reverse polarity (12 kV)	PB-DS-PB	Coaxial SL	IPA/water/acetic acid (50:50:0.1, v/v/v)	QTOF (QTOF 6540)	mAb: infliximab	
CZE	BGE: 25 mM AmAc (pH 6.8); normal polarity (30 kV), 0.5 psi	LPA	NanoSL	10 mM AmAc	Orbitrap (modified QE-EMR)	Ribosomal Proteins	
CZE	BGE: 25 mM AmAc (pH 6.8); normal polarity (30 kV), 0.725 psi	LPA	NanoSL	10 mM AmAc (pH 6.8)	QTOF (6545XT AdvanceBio LC/QTOF)	mAb: SigmaMAb, NISTmAb	
CZE	BGE: 40 mM AmAc (pH 6.8); normal polarity (15 kV), 3 psi	PAM	Sheathless porous tip	not applicable	Orbitrap (QE-EMR, UHMR, and Eclipse)	Standard proteins, GroEL	

Separation mode	Separation conditions	Coating	Interface	Sheath liquid / Make-up liquid	MS instrument	Analyte	Ref.
CZE	BGE: 40 mM AmAc (pH 6.8); normal polarity (18 kV), 2.2-15 psi	PAM	Sheathless porous tip	not applicable	Orbitrap (QE-EMR)	Synthetic and endogenous nucleosomes	
MCE (CZE)	BGE: ZipChip native Antibody kit); normal polarity (500 V/cm)	ZipChip HRN chip (native antibody kit)	ZipChip	ZipChip native Antibody kit	Orbitrap (QE Plus with extended mass Biopharma Option)	mAb: IgG4 and IgG1	
MCE (CZE)	BGE: ZipChip native Antibody kit, 4% DMSO; normal polarity (500 V/cm)	ZipChip HRN chip (native antibody kit)	ZipChip	ZipChip native Antibody kit	Orbitrap (QE Plus with extended mass Biopharma Option)	mAb: rituximab, trastuzumab and bevacizumab	
MCE (CZE)	BGE: ZipChip native Antibody kit, 4% DMSO; normal polarity (500 V/cm)	ZipChip HRN chip (native antibody kit)	ZipChip	ZipChip native Antibody kit	Orbitrap (QE)	mAb: IgG1	
MCE (CZE)	BGE: ZipChip protocol "Boosting Sensitivity for Intact Antibody Charge Variant Analysis"; normal polarity (500 V/cm)	ZipChip HRN chip (native antibody kit)	ZipChip	ZipChip native Antibody kit	Orbitrap (QE Plus with extended mass Biopharma Option)	mAb: cetuximab	

Separation mode	**Separation conditions**	**Coating**	**Interface**	**Sheath liquid / Make-up liquid**	**MS instrument**	**Analyte**	**Ref.**
MCE (CZE)	BGE: ZipChip native Antibody kit, 3.8% DMSO; normal polarity (650 V/cm)	ZipChip HRN chip (native antibody kit)	ZipChip	ZipChip native Antibody kit	Orbitrap (QE-EMR)	mAb: NISTmAb	
ACE	25 mM AmAc (pH 8.0); reverse polarity (30 kV)	PB-DS-PB	Coaxial SL	IPA/water/25 mM AmAc (pH 8.0) (25:50:25, v/v/v)	Q-TOF (micrOTOF-QII)	Bovine trypsinogen, trypsin, α-chymotrypsin, bovine aprotinin	
ACE	BGE: 50 mM AmAc (pH 6.0); normal polarity (20 kV)	PAM	Sheathless porous tip	not applicable	Orbitrap (QE-EMR)	FcRn construct, standard and engineered mAbs, Fc-only constructs	
ACE	BGE: 50 mM AmAc (pH 6.8); normal polarity (20 kV), 2 psi forward pressure	PAM	Sheathless porous tip	not applicable	FT-ICR (solariX 15 T)	FcγIIa construct, mAb A, C and E	

Table 2: Analysis of proteins using native CIEF-MS.

Separation mode	Carrier ampholyte mixture	Separation conditions	Mobilization	Coating	Interface	Sheath liquid	MS instrument	Analyte	Re
CIEF	Ampholytes: pH 3–10 (1.5% v/v) in 30:70 glycerol/water (v/v)	Anolyte: 50mM formic acid/1 mM glutamic acid (pH 2.35); catholyte: 100mM NH_3/1 mM lysine (pH 10.6); normal polarity (30 kV)	50 mbar	Bare fused silica	Coaxial SL	MeOH/water/acetic acid (50:50:1 v/v/v)	Single quadrupole (Series 1100 MSD)	Ribonuclease A, a-chymotrypsinoge A type II, myoglobin, carbonic anhydrase II, b-lactoglobulin A, trypsin inhibitor	
CIEF	Ampholytes: pH 3–10 (1.5% v/v) and pH 9–11 (1% v/v) in 40/60 (v/v) glycerol/water	Anolyte: 50 mM formic acid/1 mM glutamic acid (pH 1.9); catholyte: 100 mM NH_3/1 mM arginine (pH 11.3); normal polarity (30 kV);	50 mbar, 30 kV	LPA	Coaxial SL	10 mM AmAc (pH 5.0)	Ion trap (Esquire 3000+)	Recombinant wild-type IFN-γ, single and deletion mutants	

Separation mode	Carrier ampholyte mixture	Separation conditions	Mobilization	Coating	Interface	Sheath liquid	MS instrument	Analyte	Re
CIEF	For SA/CA Ampholytes: pH 3-10 and pH 5-8 (1.5% v/v in total) in 10 mM AmAc and 15% glycerol (pH 6.7); For ADC Ampholytes: 0.75% (v/v) pH 3-10 and 8-10.5 with a ratio of 1:2	Anolyte: 0.1% (v/v) formic acid (FA), 15% glycerol (pH 3.0); catholyte: 10 mM AmBicarb, 15% glycerol, (pH 10.0); normal polarity (30 kV);	10 mbar, 20 kV	LPA	Nano SL	10 mM AmAc, 10% (v/v) ACN (pH 5.0)	Q-TOF (6545XT)	Recombinant streptavidin (SA), carbonic anhydrase II (CA), interchain cysteine-linked antibody-drug conjugate (ADC)