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Nguyet Thuy Tran
Myriam Taverna *Editors*

Capillary Electrophoresis of Proteins and Peptides

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Capillary Electrophoresis of Proteins and Peptides

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Edited by

Nguyet Thuy Tran and Myriam Taverna

Institut Galien Paris-Sud, UMR 8612, Protein and Nanotechnology in Analytical Science (PNAS), CNRS, Univ. Paris-Sud, Université Paris-Saclay, Châtenay-Malabry, France

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Editors
Nguyet Thuy Tran
Institut Galien Paris-Sud, UMR 8612, Protein and Nanotechnology in Analytical Science (PNAS), CNRS
Univ. Paris-Sud, Université Paris-Saclay
Châtenay-Malabry, France

Myriam Taverna
Institut Galien Paris-Sud, UMR 8612, Protein and Nanotechnology in Analytical Science (PNAS), CNRS
Univ. Paris-Sud, Université Paris-Saclay
Châtenay-Malabry, France

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Preface

Peptides and proteins represent an important class of biomolecules which play essential roles in living organisms by regulating or controlling many biological processes. At the same time, they display very diverse functions, acting as hormones, substrates, neurotransmitters, immunomodulators, enzymes, coenzymes, receptor ligands, transporters, and toxins. Proteins and peptides are also very important biopharmaceuticals, and their therapeutic use requires an extensive structural characterization as well as multiple quality controls of these complex drugs.

This book provides a comprehensive survey of recent developments and applications of high performance capillary electrophoresis in the field of protein and peptide analysis. The focus is given to the analysis of intact proteins. (Glyco)proteins can also be analyzed as fragments (peptides, glycopeptides, oligosaccharides, monosaccharides) after their enzymatic or chemical cleavage, and this would represent another set of analytical strategies and topics that have not been considered in this book. This book covers different modes of capillary electrophoresis (CE) useful for protein and peptide analysis, CZE, CIEF, ACE, CGE, and different types of application such as the quality control of therapeutic proteins and monoclonal antibodies, clinical analyses of chemokines in tissues, qualitative and quantitative analysis of vaccine proteins, and determination of binding constants in complexes involving peptides or proteins.

CE is a powerful separation technique; it brings speed, high resolution, automation, and low consumption of samples and buffers. However, the separation of proteins by CE is often complicated by their tendency to adsorb onto the negatively charged surface of fused silica capillaries. This occurs primarily via coulombic interaction but can rapidly get amplified by the unfolding of the adsorbed proteins which can in turn participate, through a cooperative effect, to adsorb other proteins involving at this stage all kind of molecular interactions (hydrophobic, dipolar, hydrogen bounding, etc.). Different strategies, with different levels of success, can be employed to circumvent these issues. They may rely on simply working at extreme pHs (acidic or alkaline) or increasing ionic strengths of the buffers to more drastic solutions that entail dynamic or permanent capillary coatings.

The first part is devoted to detection methods employed in CE for proteins and peptides, a topic as important as the separation itself. Proteins can be easily detected with UV detection in CE; however, to achieve more sensitive detection, laser-induced fluorescence detection may be preferable. Even if proteins have tryptophan residues that possess intrinsic fluorescence properties, most of the sensitive applications require the derivatization of peptides or proteins with fluorophore dyes. Another way to achieve high sensitivity is to use mass spectrometry (MS) as a detector. The number of applications using the coupling of CE to MS has significantly increased these last 10 years. CE-MS indeed combines the high separation efficiency of CE with the possibility of mass detection and analyte characterization through MS-MS. Besides, the interfacing technology has considerably evolved proposing, at present configurations with or without addition of sheath liquid, to maintain the electrical continuity required for the electrophoretic separation. It remains that not all CE separation conditions are compatible with MS, but researchers are progressing, trying to

push the current limits of CE-MS. CIEF combined with MS is possible, while this coupling presents major challenges as the compatibility between the separation medium, which contains anticonvective gel and ampholytes, and MS detector.

The second part provides the readers with the latest breakthroughs and improvements in CE. This part encompasses many contributions showing that CE is an evolving technique which is still very active in providing innovations and new solutions to circumvent protein adsorption, to increase detection sensitivities or specificities. The recent advances have mainly been focused into two directions: the sample pretreatments online to the separation and the integration of electrophoretic processes into microchips. Sample treatment is often required for real biological samples either to eliminate interfering compounds, matrices, or to enrich the sample with the protein present at a too low concentration if body fluid or tissues are studied. Monoliths which can be easily synthetized into capillaries are among the solid supports that rank amongst the most adapted for online sample pretreatments.

Part 3 highlights different recent applications in the field of quality control of therapeutic proteins. This part is fully illustrated by protocols dealing with recombinant proteins such as growth hormone, insulin, plasma-derived proteins such as human serum albumin as well as monoclonal antibodies. Those applications are proposed for formulated pharmaceutical preparations, and this can complicate the analytical development of the CE method due to the presence of specific excipients in those formulations aimed at protecting the active proteins from degradation or ensuring a longer or controlled release of the drug.

Finally, Part 4 illustrates quite specific applications of CE analysis in the field of vaccine proteins, or peptide/alkali metal ion complexes, showing that CE can be applied to very different areas in health and therapeutics and even to give more insight on the way proteins are acting or interacting.

This book is useful for a wide audience, including researchers, technicians, and students; it can also be a reference for experienced researchers as well as for beginners and newcomers in this field. Indeed, besides specific methods fully detailed, several important principles related to protein analyses by CE are briefly noted in several chapters (separation modes, capillary coatings, chemical and physical protein degradation ways, CE modes for intact glycoprotein analysis, methods for coupling solid phase extraction to CE, methods for pre-capillary, in-capillary, or post-capillary derivatizing proteins, CE-MS coupling).

As editor and co-editors of this book, we would like to thank all the chapter contributors who made the editing of this book possible by their excellent work covering quite exhaustively the current and most active topics of CE for peptides and proteins.

Châtenay-Malabry, France

Nguyet Thuy Tran
Myriam Taverna

Contents

Contributors

Ahmad Amini • *Medical Products Agency (MPA), Uppsala, Sweden; Division of Analytical Pharmaceutical Chemistry, Biomedical Centre, Uppsala University, Uppsala, Sweden*

Fernando Benavente • *Department of Analytical Chemistry, University of Barcelona, Barcelona, Spain*

Sylvie Boucher • *Centre for Biologics Evaluation, Biologics and Genetic Therapies Directorate, Health Canada, Ottawa, ON, Canada*

Audrey Boutonnet • *Picometrics Technologies, Labège, France*

David D.Y. Chen • *Department of Chemistry, University of British Columbia, Vancouver, BC, Canada*

Marcella Chiari • *Istituto di Chimica del Riconoscimento Molecolare, CNR, Milano, Italy*

François Couderc • *Laboratoire des IMRCP, Université Paul Sabatier, Université de Toulouse, Toulouse, France*

Marina Cretich • *Istituto di Chimica del Riconoscimento Molecolare, CNR, Milano, Italy*

Fanny d'Orlyé • *Chimie ParisTech, PSL Research University Unité de Technologies Chimiques et Biologiques pour la Santé, Paris, France; INSERM, Unité de Technologies Chimiques et Biologiques pour la Santé (n° 1022), Paris, France; CNRS, Unité de Technologies Chimiques et Biologiques pour la santé UMR 8258, Paris, France; Université Paris Descartes, Sorbonne Paris Cité, Unité de Technologies Chimiques et Biologiques pour la Santé, Paris, France*

Elena Domínguez-Vega • *Division of BioAnalytical Chemistry, VU University, Amsterdam, The Netherlands*

Vincent Ecochard • *IPBS, Université Paul Sabatier, Université de Toulouse, Toulouse, France*

Changlu Gao • *School of Marine Science and Technology, Harbin Institute of Technology at Weihai, Shandong, China*

Estela Giménez • *Department of Analytical Chemistry, University of Barcelona, Barcelona, Spain*

Michel Girard • *Centre for Biologics Evaluation, Biologics and Genetic Therapies Directorate, Health Canada, Ottawa, ON, Canada*

Melissa Hamm • *Merck Research Laboratories, Vaccine Analytical Development, West Point, PA, USA*

Rob Haselberg • *Division of BioAnalytical Chemistry, VU University, Amsterdam, The Netherlands*

Bo Hu • *School of Marine Science and Technology, Harbin Institute of Technology at Weihai, Shandong, China*

Emmanuel Jaccoulet • *Institut Galien Paris Sud, UMR 8612, Protein and Nanotechnology in Analytical Science (PNAS), CNRS, Univ. Paris-Sud, Université Paris-Saclay, Châtenay-Malabry, France*

Caitlyn A.G. De Jong • *Department of Chemistry, University of British Columbia, Vancouver, BC, Canada*

Heather Kalish • *Microanalytical Immunochemistry Unit, Biomedical Engineering and Physical Science Shared Resource, National Institute of Biomedical Imaging and Bioengineering, National Institutes of Health, Bethesda, MD, USA*
Anita Kane • *Centre for Biologics Evaluation, Biologics and Genetic Therapies Directorate, Health Canada, Ottawa, ON, Canada*
Takashi Kaneta • *Department of Chemistry, Graduate School of Natural Science and Technology, Okayama University, Okayama, Japan*
Václav Kašička • *Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Prague, Czech Republic*
Catherine Lancaster • *Merck Research Laboratories, Vaccine Analytical Development, West Point, PA, USA*
Alexis K. Lee • *Department of Chemistry, University of British Columbia, Vancouver, BC, Canada*
John W. Loughney • *Merck Research Laboratories, Vaccine Analytical Development, West Point, PA, USA*
Anne-Lise Marie • *Institut Galien Paris Sud, UMR 8612, Protein and Nanotechnology in Analytical Science (PNAS), CNRS, Univ. Paris-Sud, Université Paris-Saclay, Châtenay-Malabry, France*
Shane McMohan • *Microanalytical Immunochemistry Unit, Biomedical Engineering and Physical Science Shared Resource, National Institute of Biomedical Imaging and Bioengineering, National Institutes of Health, Bethesda, MD, USA*
Silvia Medina-Casanellas • *Department of Analytical Chemistry, University of Barcelona, Barcelona, Spain*
Meriem Mokaddem • *Chimie ParisTech, PSL Research University Unité de Technologies Chimiques et Biologiques pour la Santé, Paris, France; INSERM, Unité de Technologies Chimiques et Biologiques pour la Santé (n° 1022), Paris, France; CNRS, Unité de Technologies Chimiques et Biologiques pour la santé UMR 8258, Paris, France; Université Paris Descartes, Sorbonne Paris Cité, Unité de Technologies Chimiques et Biologiques pour la Santé, Paris, France*
Elena Domínguez-Vega • *Division of BioAnalytical Chemistry, VU University, Amsterdam, The Netherlands*
Sabine Neuberger • *Department of Chemistry, Aalen University, Aalen, Germany*
Christian Neusüß • *Department of Chemistry, Aalen University, Aalen, Germany*
Terry M. Phillips • *Microanalytical Immunochemistry Unit, Biomedical Engineering and Physical Science Shared Resource, National Institute of Biomedical Imaging and Bioengineering, National Institutes of Health, Bethesda, MD, USA; National Institute of Biomedical Imaging and Bioengineering, Washington DC, USA*
Isabelle Le Potier • *Institut Galien Paris Sud, UMR8612, Protein and Nanotechnology in Analytical Science (PNAS), CNRS, Université Paris-Sud, Université Paris-Saclay, Châtenay-Malabry, France*
Wei Qiao • *School of Marine Science and Technology, Harbin Institute of Technology at Weihai, Shandong, China*
Angelina Rafai • *Solvias AG, Kaiseraugst, Switzerland*
Jessica Risley • *Department of Chemistry, University of British Columbia, Vancouver, BC, Canada*
Serge Rudaz • *School of Pharmaceutical Sciences, University of Geneva, University of Lausanne, Geneva, Switzerland*

Richard R. Rustandi • *Merck Research Laboratories, Vaccine Analytical Development, West Point, PA, USA*
Victoria Sanz-Nebot • *Department of Analytical Chemistry, University of Barcelona, Barcelona, Spain*
Julie Schappler • *School of Pharmaceutical Sciences, University of Geneva, University of Lausanne, Geneva, Switzerland*
Claire Smadja • *Institut Galien Paris Sud, UMR 8612, Protein and Nanotechnology in Analytical Science (PNAS), CNRS, Univ. Paris-Sud, Université Paris-Saclay, Châtenay-Malabry, France*
Laura Sola • *Istituto di Chimica del Riconoscimento Molecolare, CNR, Milano, Italy*
Govert W. Somsen • *Division of BioAnalytical Chemistry, VU University, Amsterdam, The Netherlands*
Sille Štěpánová • *Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Prague, Czech Republic*
Xiuhua Sun • *School of Marine Science and Technology, Harbin Institute of Technology at Weihai, Shandong, China*
Myriam Taverna • *Institut Galien Paris Sud, UMR 8612, Protein and Nanotechnology in Analytical Science (PNAS), CNRS, Univ. Paris-Sud, Université Paris-Saclay, Châtenay-Malabry, France*
Nguyet Thuy Tran • *Institut Galien Paris Sud, UMR 8612, Protein and Nanotechnology in Analytical Science (PNAS), CNRS, Univ. Paris-Sud, Université Paris-Saclay, Châtenay-Malabry, France*
Anne Varenne • *Chimie ParisTech, PSL Research University Unité de Technologies Chimiques et Biologiques pour la Santé, Paris, France; INSERM, Unité de Technologies Chimiques et Biologiques pour la Santé (n° 1022), Paris, France; CNRS, Unité de Technologies Chimiques et Biologiques pour la santé UMR 8258, Paris, France; Université Paris Descartes, Sorbonne Paris Cité, Unité de Technologies Chimiques et Biologiques pour la Santé, Paris, France*
Elena Domínguez-Vega • *Division of BioAnalytical Chemistry, VU University, Amsterdam, The Netherlands*
Huaixin Wang • *School of Marine Science and Technology, Harbin Institute of Technology at Weihai, Shandong, China*
Edward Wellner • *Retired from National Institutes of Health, Bethesda, MD, USA*
Shuai Sherry Zhao • *Department of Chemistry, University of British Columbia, Vancouver, BC, Canada*

Chapter 1

Chemical and Instrumental Approaches for Capillary Electrophoresis (CE)–Fluorescence Analysis of Proteins

Isabelle Le Potier, Audrey Boutonnet, Vincent Ecochard, and François Couderc

Abstract

Capillary electrophoresis (CE) coupled to fluorescence detection is an invaluable technique for the quantitative analysis of proteins of interest in the field of clinical diagnosis and quality control of novel biotechnology products. The various chemical and instrumental approaches that have been reported to carry out such sensitive analysis are described in this paper. To illustrate the contribution of CE to the analysis of therapeutic proteins, a detailed protocol for impurities profiling of a recombinant antibody sample using CE-LEDIF is given.

Key words Capillary electrophoresis, Laser-induced fluorescence, Light-emitting diodes, Proteins, Labeling procedures

1 Introduction

Capillary electrophoresis (CE) is a powerful separation technique for proteins due to its high efficiency, fast separation speed, and low sample consumption [1–3]. Among the commercial detection modes developed for CE, fluorescence detection is one of the most attractive to enhance detection sensitivity of proteins due to the low detection limits that can be attained. In the field of clinical diagnosis and clinical chemical analysis, CE coupled to laser-induced fluorescence (CE-LIF) is therefore an analytical technique particularly adapted to analyze biological samples, where micro- and nanomolar protein concentrations are common [4, 5]. This technique is also suitable for quality control analysis of recombinant therapeutic proteins allowing their characterization in terms of heterogeneity, glycosylation, impurity profiling, etc. [6].

1.1 Labeling Reagents

To carry out such sensitive analysis, labeling reagents are generally used to confer fluorescent properties on proteins since many of them do not exhibit a sufficient native fluorescence due to the lack

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or low quantity of amino acids exhibiting UV-absorbing moieties (i.e., Trp, Tyr, and/or Phe residues). These reagents should have several characteristics: (1) production of a fluorescent derivative with high fluorescence quantum yield showing an excitation wavelength that matches with available light source, (2) low background signal and limited production of side products, (3) reproducible reaction and fast reaction rate with the analyte, and (4) stability of the fluorescent adducts.

The labeling reagents used for CE-LIF of proteins can be classified into two groups: (1) covalent dyes which covalently bind the protein moiety and (2) dyes which interact by affinity with the protein to form a strongly fluorescent protein–dye complex [7, 8].

Covalent labeling, which is by far the most common approach employed, may be obtained by reaction of the dyes with the available functional groups of proteins: amino, thiol, and carboxylate functions. Because the carboxylate group must be activated before derivatization, this approach is not frequently employed compared to the two others. The thiol group is highly reactive but the low abundance of Cys residues limits the wide use of thiol-reactive dyes for protein detection. Therefore, among all the covalent dyes reported for protein labeling, amine-reactive dyes such as fluorescein- and rhodamine-based dyes are the most often used to label primary amino groups such as the α-NH_2 of the N-terminus and the aliphatic ε-NH_2 of Lys residue and, to a lesser extent, the secondary amino functional groups of Arg and His residues [7–9]. However, the presence of these multiple amino functions in protein can complicate the electropherograms because of the production of various forms of derivatized analytes bearing a different number of covalently attached tags (multiple labeling). This problem can be overcome by using non-covalent dyes.

The molecules used for non-covalent labeling, which have been for most of them initially developed for the in-gel staining of SDS-PAGE, are able to bind to proteins through electrostatic and/or hydrophobic interactions and their use for CE-LIF of protein can be an attractive alternative to covalent labeling. The labeling of proteins with these non-covalent reagents such as nano orange, sypro orange, and sypro red has been successfully applied to various CE separation modes [7, 8, 10, 11]. However, this approach can lead to low peak efficiencies owing to the slow kinetics of the association equilibrium between the dye and the protein which is a limiting factor for their wide use [12].

Among the different fluorophores reported to label polypeptides, it must be emphasized that fluorogenic dyes such as naphthalene-2,3-dicarboxaldehyde (NDA), 3-(4-carboxybenzoyl)-2-quinoline-carboxaldehyde (CBQCA), and 3-(2-furoyl) quinoline-2-carboxyaldehyde (FQ) are particularly useful for CE-LIF of proteins [7–9, 13–15]. These non-fluorescent reagents do not exhibit any fluorescence properties when they are not linked

to the analytes, but form a strongly fluorescent derivative when interacting through covalent or non-covalent interactions with the protein. Their use in place of a fluorescent dye is attractive because it reduces the background signal by several orders of magnitude.

1.2 Labeling Modes

The labeling of proteins for CE-LIF analyses can be accomplished by three different labeling procedures: pre-, in-, and post-capillary ones [7, 8, 16].

Pre-capillary derivatization procedure, in which the labeling is performed prior to the electrokinetic separation, is the most frequently used one for labeling. The reason for this is that all types of reagents, both fluorescent and fluorogenic, can be employed. Moreover, it allows a high flexibility in reaction conditions (extreme conditions such as high pH, high temperatures, or long reaction times are possible). The excess of labeling reagent, which can make the detection of derivatized protein at low concentration difficult, may be removed by sample pre-treatment such as solid–liquid extraction [16].

In-capillary procedure consists of developing the labeling reaction inside the separation capillary by promoting the mixing of the protein with the reagent. In-capillary labeling, which may be classified into three groups: zone-passing (1), at-inlet (2), and throughout-capillary (3) techniques, is also popular in CE because it avoids complicated set-ups and it is adapted to very small sample volumes since dilution is reduced to the minimum. (1) Briefly, the zone-passing method is based on labeling in the middle of the capillary by passing either sample or reagent zone through the other under an electric field applied just after the introduction of the reagent and the sample solutions into the capillary [16, 17]. (2) In the at-inlet technique, the sample and reagent solutions are introduced at the inlet of the capillary either by tandem or sandwich mode. The reactants are mixed by diffusion and allowed to react for a specified time before applying the separation voltage to transport the labeled derivatives to the detector [16, 18]. While zone-passing method is well adapted to fast reaction rates, at-inlet method is compatible with slow reactions which take several minutes to occur completely. (3) In the throughout-capillary technique, the sample solution is introduced at the inlet of the capillary previously filled with a running buffer which contains the labeling reagent. When the electric field is applied, the analyte migrates and mixes with the reagent allowing the labeling to occur. This mode is a suitable approach for non-covalent labeling of protein using a fluorogenic reagent [12, 16].

Post-capillary labeling procedures, in which labeling is performed after the electrokinetic separation, are much less employed compared to pre- and in-capillary techniques [13, 16]. This is mainly due to the lack of commercially available devices but also because it requires short reaction times to maintain efficiency and fluorogenic dyes to limit the background signal.

1.3 Instrumentation

Fluorescence measurement is generally based on the use of labeling reagents that absorb in the UV–visible wavelength range because of the available light sources. Lasers are commonly used as light source because laser light is highly collimated which enables the light to be focused onto the small diameter of the capillary. Laser-induced fluorescence (LIF) is far more sensitive than conventional fluorescence induced by arc lamps as light source [19, 20]. Gas lasers and more recently diode lasers are commercially available and compatible with the fluorescence detectors of CE commercial instruments. Most of them excite fluorescent derivatives in the 350–650 nm wavelength range, although deep-UV and near-infrared excitation lasers are also available. Light-emitting diodes (LEDs) represent an attractive alternative light source to lasers. LEDs produce a very energy-efficient monochromatic light and allow a wide range of wavelengths to better match the maximum wavelength for the fluorescence of the fluorescent derivative. They are also less expensive, consume less energy, and are more stable compared to conventional lasers [21, 22].

An exhaustive list of fluorophores employed in CE to label proteins can be found in several reviews [7, 8, 16, 23]. The chemical and spectral properties of these reagents (i.e. fluorescent or fluorogenic character, maximum excitation and emission wavelengths, mechanism and rate of the labeling reaction, etc.) and the labeling procedures previously reported in the literature are described in detail in these comprehensive papers dedicated to CE-LIF of amino acids, peptides, and proteins. Readers can also refer to these reviews for detailed information concerning the instrumentation for CE-LIF and CE coupled to LED-induced fluorescence (CE-LEDIF).

In this study, we describe a method to analyze the impurities of an IgG sample using CE-LEDIF. The analysis of recombinant IgG is commonly performed using CE-LIF [24, 25] and CE-UV [26]. Since the detection of low level of impurities is difficult via CE-UV, CE-LIF was used to increase sensitivity. The common availability of the Argon ion laser (488 nm) had led to the use of three different labels for IgG, 5-carboxytetramethylrhodamine succinimidyl ester (TAMRA) [24, 25], FQ in the presence of cyanide ion (CN^-) as a nucleophile [14], and fluorescein isothiocyanate (FITC) [22]. The use of FQ is a good alternative, because this dye is a fluorogenic dye which does not fluoresce when it did not react with a primary amine. It was demonstrated using mass spectrometric studies that the obtained derivative is not unique and is composed of several covalent adducts of FQ with the protein (three to eight FQ molecules can be fixed to the antibody). The high number of attached dye molecules increases the fluorescence of the labeled compound, but the multi-tagging of the analytes may cause confusing results between the different FQ adducts and isoforms of the antibodies and the impurities [27].

FQ has been extensively used for CE-LIF IgG analysis, but it has never been used with fluorescent detection induced by a LED. Moreover, as indicated above, the LED has a higher light power than a conventional laser and a very small space occupancy. We have previously shown that the baseline of the LED is much more stable than the one obtained with a laser [28]. In this study, we will present how to analyze the impurities in a IgG sample derivatized using FQ and a LED as excitation source of a CE-fluorescence detector.

2 Materials

Prepare all solutions using ultrapure water (unless indicated otherwise) and analytical grade reagents. Prepare and store all reagents at room temperature (unless indicated otherwise).

2.1 FQ Labeling of IgG

1. 0.1 M citrate-phosphate (pH 6.5) buffer (buffer A): Add 1.6 mL of the Sigma citrate-phosphate buffer (ref. 2851) to 8.4 mL of deionized water. Mix and adjust pH with 1 M NaOH. Store this solution at 4 °C.
2. 1 mM FQ solution: Dissolve 10 mg FQ (from Invitrogen, ref A10192) in 1 mL of methanol to obtain a 40 mM FQ stock solution (*see* **Note 1**). Mix 2.5 µL of FQ stock solution in 97.5 µL of deionized water.
3. 10% w/v sodium dodecyl sulfate (SDS) solution: Dissolve 1 g of SDS (from Aldrich, ref 86201-0) in 10 mL of deionized water. Store this solution at room temperature, it can be stored up to 1 week.
4. 100 mM potassium cyanide (KCN) solution: Dissolve 6.5 mg of KCN (from Aldrich, ref 20781-0) in 1 mL of ultrapure water. Store this solution at room temperature up to 1 week.
5. 100 mM *N*-ethylmaleimide (NEM) solution: Dissolve 125 mg of NEM (from Aldrich, ref E3876) in 10 mL of ultrapure water. Store this solution at room temperature up to 1 week.
6. IgG solution at 2 mg/mL: 2 mg of IgG from human serum (from Sigma, I4506) are precisely weighed and dissolved in 1 mL of buffer A. Store this solution at –20 °C.
7. To reduce the cystines in the sample, 3 µL of 2-mercaptoethanol are added to 30 µL of IgG sample at 2 mg/mL. The sample is heated at 70 °C for 5 min.

2.2 Operational Qualification Test of CE System

1. 25 mM carbonate/bicarbonate solution: Dissolve the content of one buffer capsule (from Sigma ref C-3041) in 200 mL of deionized water.
2. FITC solution at 39 ng/L: Dissolve 3.90 mg FITC (from Sigma ref F-4274) in 10 mL DMF (stock solution). Dilute

FITC stock solution with two 1/100 dilutions in 25 mM carbonate/bicarbonate buffer (first 1/100 dilution: 10 µL of FITC stock solution in 990 µL of carbonate/bicarbonate buffer; idem second 1/100 dilution, vortex after each dilution).

2.3 Sensitivity Checking

1. IgG solution at 4 mg/mL: Weigh precisely 4 mg of IgG and dissolve in 1 mL of buffer A. Store this solution at −20 °C.
2. Trypsin inhibitor solutions at concentrations ranging from 20 to 200 µg/mL: Dissolve 1 mg of trypsin inhibitor (from Sigma ref T9767) in 1 mL of buffer A (stock solution). Dilute 1 mg/mL stock solution in buffer A to obtain the working solutions at 200, 80, 40, and 20 µg/mL.
3. For 0% contaminant level, add 20 µL of buffer A to 20 µL of IgG solution at 4 mg/mL;

   For 0.5% contaminant level, add 20 µL of 20 µg/mL solution of trypsin inhibitor to 20 µL of IgG solution at 4 mg/mL;

   For 1% contaminant level, add 20 µL of 40 µg/mL solution of trypsin inhibitor to 20 µL of IgG solution at 4 mg/mL;

   For 2% contaminant level, add 20 µL of 80 µg/mL solution of trypsin inhibitor to 20 µL of IgG solution at 4 mg/mL;

   For 5% contaminant level, add 20 µL of 200 µg/mL solution of trypsin inhibitor to 20 µL of IgG solution at 4 mg/mL.

3 Methods

3.1 FQ Labeling of IgG

1. Add 35 µL of IgG solution at 2 mg/mL, 7 µL of SDS solution at 10% (w/v), 4 µL of 100 mM NEM solution, 0.5 µL of 100 mM KCN solution, 25 µL of 1 mM FQ solution.
2. Mix the solution and heat at 75 °C for 5 min.
3. Place the Eppendorf in ice for 5 min.
4. Add 30 µL of SDS solution at 10% (w/v) in the Eppendorf and mix the solution.

3.2 CE Method

Agilent Technologies Model 7100 Capillary Electrophoresis system (Waldbronn, Germany) equipped with a LIF detector (Picometrics Technologies, Toulouse, France). Experiments were performed using a LED 480 nm.

Fused-silica capillaries (Polymicro Technologies, Phoenix, AZ) of 50 µm ID and 375 µm OD with a total length of 33 cm and an effective length of 19 cm (*see* **Note 2**).

3.2.1 Conditioning of a New Capillary

1. Flush the uncoated capillary with 1 M NaOH (*see* **Note 3**) for 3 min at 1 bar and with ultrapure water for 3 min at 1 bar.
2. Check that liquid is present in the waste.

3.2.2 Operational Qualification Test of the CE System

1. Flush the capillary with 39 ng/L FITC solution (3 min at 1 bar): The fluorescence value must exceed 5 RFU.
2. Flush the capillary with 1 M NaOH solution (3 min at 1 bar) and deionized water (3 min at 1 bar). The fluorescence level must decrease to less than 3 RFU. This procedure ensures that the capillary is not blocked.
3. Flush the capillary with SDS-MW gel buffer for 15 min at 6 bar (*see* **Notes 4–6**). Apply −16 kV for a period of 5 min. Check that the current is stable (35 μA). If it is not, restart the flush with SDS-MW gel buffer.

3.2.3 Separation Method

1. Flush at 6 bar the capillary with the following sequence: 3 min 1 M NaOH, 3 min 0.1 M HCl, 3 min deionized water and 10 min with SDS-MW gel buffer.
2. Inject the sample at −10 kV for 10 s (*see* **Notes 7** and **8**).
3. Apply voltage at −16 kV for 40 min.
4. Set the capillary cassette temperature at 40 °C.
5. Set the rise time of the detector at its minimum value of 0.5 s to electronically filter the high-frequency noise.

3.3 Sensitivity Checking with Trypsin Inhibitor

The limit of detection (LOD) is determined with spiked contaminants (trypsin inhibitor, 28 kDa) of known concentration: 0, 0.5, 1, 2, and 5% (w/w) levels.

The CE-LEDIF analysis of IgG solutions spiked with trypsin inhibitor is presented in Fig. 1.

The S/N ratio at 0.5% (w/w) is 65. The extrapolation to a S/N ratio of 3 shows a limit of detection at 0.023% (w/w).

The percentage of relative standard deviation (% RSD) on 5 analyses was 1.87% for the peak area and 0.19% for the migration time of IgG.

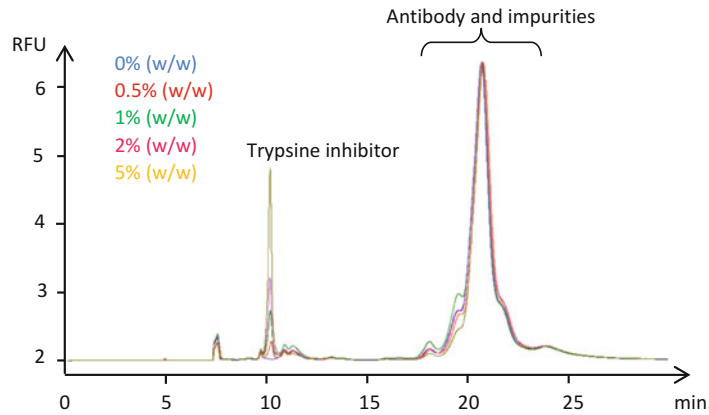

Fig. 1 CE-LEDIF analysis of human IgG labeled with FQ with a 28 kDa protein spike (trypsin inhibitor) at 5, 2, 1, 0.5, and 0%

3.4 Some Impurities Identification in IgG

Figure 2a presents the separation of the IgG, where we can see the presence of some impurities, to identify these impurities, the sample is reduced.

The different peaks obtained in Fig. 2b can be attributed to a light chain, a heavy chain, and a peak for the deglycosylated heavy chain. In consequence, these peaks can be identified in the derivatized IgG, the major peak being the intact IgG. At higher migration times, aggregates are observed. At lower migration time (in the order of decreasing migration times) the mono unglycosylated IgG, the di unglycosylated IgG, the heavy chain, the unglycosylated heavy chain, and the light chain are observed. A peak at 7 min migration time is identified as fluoresceinamine, the degradation product of FITC.

4 Notes

1. The FQ solution at 40 mM is stored at −20 °C with aliquot of 25 μL.
2. Clean the window of the capillary with HPLC grade methanol and lint-free lens tissue. All residue and fingerprints must be removed to ensure minimal dispersion. Check that the window is clean with binocular microscope or microscope.
3. To prepare the solution of 1 M NaOH: Weigh 4.00 g of pellets and dissolve them in 100 mL of distilled water. Mix this solution for 5 min.
4. The SDS-MW gel buffer is stored at room temperature.
5. To flush the capillary with SDS-MW gel buffer, the high pressure (6 bars) is used. An external pressure is connected to the Agilent Technologies CE.
6. The SDS-MW gel buffer is viscous. The electrodes and prepunchers should be cleaned with water and methanol every week.

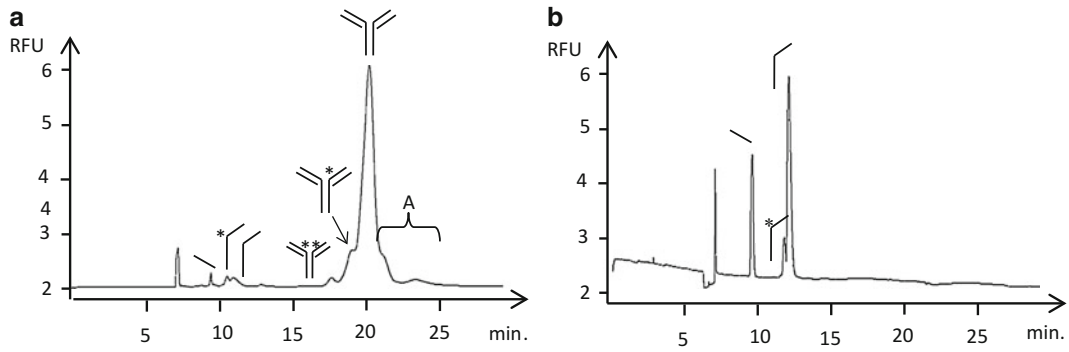

Fig. 2 CE-LEDIF analysis of human IgG labeled with FQ. Non-reduced (**a**) and reduced protein sample (**b**). Light chain is presented as a straight tilted line, heavy chain as a bent line, nonglycosylated heavy chain, A aggregates

7. The effective length of the capillary is 19 cm. So in this case, the injection is on the outlet side.
8. When using microvials for samples, make sure there are no air bubbles in the vials. Current problems may occur if air bubbles are injected.

References

1. Strege MA, Lagu AL (eds) (2004) Capillary Electrophoresis of Proteins and Peptides. Methods in Molecular Biology, Vol. 276, (Series Editor Walker JM). Humana Press, Totowa, NJ, pp 332
2. El Rassi Z (2010) Electrophoretic and electrochromatographic separation of proteins in capillaries: an update covering 2007-2009. Electrophoresis 31:174–191
3. Righetti PG, Sebastiano R, Citterio A (2013) Capillary electrophoresis and isoelectric focusing in peptide and protein analysis. Proteomics 13:325–340
4. Szoko E, Tabi T (2010) Analysis of biological samples by capillary electrophoresis with laser induced fluorescence detection. J Pharm Biomed Anal 53:1180–1192
5. Mesbah K, Verpillot R, de L'escaille F et al (2013) Contribution of CE to the analysis of protein or peptide biomarkers. Methods Mol Biol 984:167–190
6. Zhao SS, Chen DD (2014) Applications of capillary electrophoresis in characterizing recombinant protein therapeutics. Electrophoresis 35:96–108
7. Lacroix M, Poinsot V, Fournier C et al (2005) Laser-induced fluorescence detection schemes for the analysis of proteins and peptides using capillary electrophoresis. Electrophoresis 26:2608–2621
8. García-Campaña AM, Taverna M, Fabre H (2007) LIF detection of peptides and proteins in CE. Electrophoresis 28:208–232
9. Korchane S, Pallandre A, Przybylski C et al (2014) Derivatization strategies for CE-LIF analysis of biomarkers: toward a clinical diagnostic of familial transthyretin amyloidosis. Electrophoresis 35:1050–1059
10. Harvey MD, Bablekis V, Banks PR et al (2001) Utilization of the non-covalent fluorescent dye, NanoOrange, as a potential clinical diagnostic tool. Nanomolar human serum albumin quantitation. J Chromatogr B 754:345–356
11. Chiu TC, Lin YW, Huang CC et al (2003) A simple, rapid, and sensitive method for analysis of SYPRO Red labeled sodium dodecyl sulfate-protein complexes by capillary electrophoresis with laser-induced fluorescence. Electrophoresis 24:1730–1736
12. Descroix S, Le Potier I, Niquet C et al (2005) In-capillary non-covalent labeling of insulin and one gastrointestinal peptide for their analyses by capillary electrophoresis with laser-induced fluorescence detection. J Chomatogr A 1087:203–209
13. Ye M, Hu S, Quigley WW et al (2004) Post-column fluorescence derivatization of proteins and peptides in capillary electrophoresis with a sheath flow reactor and 488 nm argon ion laser excitation. J Chromatogr A 1022:201–206
14. Michels DA, Brady LJ, Guo A et al (2007) Fluorescent derivatization method of proteins for characterization by capillary electrophoresis-sodium dodecyl sulfate with laser-induced fluorescence detection. Anal Chem 79:5963–5971
15. Asermely KE, Broomfield CA, Nowakowski J et al (1997) Identification of a recombinant synaptobrevin-thioredoxin fusion protein by capillary zone electrophoresis using laser-induced fluorescence detection. J Chromatogr B Biomed Sci Appl 695:67–75
16. Underberg WJ, Waterval JC (2002) Derivatization trends in capillary electrophoresis: an update. Electrophoresis 23:3922–3933
17. Le Potier I, Franck G, Smadja C et al (2004) In-capillary derivatization approach applied to the analysis of insulin by capillary electrophoresis with laser-induced fluorescence detection. J Chromatogr A 1046:271–276
18. Taga A, Honda S (1996) Derivatization at capillary inlet in high-performance capillary electrophoresis Its reliability in quantification. J Chromatogr A 742:243–250
19. Gooijer C, Mank AJP (1999) Laser spectroscopy in analytical chemistry: light on the next millennium. Anal Chim Acta 400:281–295
20. Johnson ME, Landers JP (2004) Fundamentals and practice for ultrasensitive laser-induced fluorescence detection in microanalytical systems. Electrophoresis 25:3513–3527
21. Zhao S, Yuan H, Xiao D (2006) Optical fiber light-emitting diode-induced fluorescence detection for capillary electrophoresis. Electrophoresis 27:461–467

22. Rodat A, Kalck F, Poinsot V et al (2008) An ellipsoidal mirror for detection of laser-induced fluorescence in capillary electrophoresis system: applications for labelled antibody analysis. Electrophoresis 29:740–746
23. Poinsot V, Carpéné MA, Bouajila J et al (2012) Recent advances in amino acid analysis by capillary electrophoresis. Electrophoresis 33:14–35
24. Hunt G, Nashabeh W (1999) Capillary electrophoresis sodium dodecyl sulfate nongel sieving analysis of a therapeutic recombinant monoclonal antibody: a biotechnology perspective. Anal Chem 71:2390–2397
25. Salas-Solano O, Tomlinson B, Du S et al (2006) Optimization and validation of a quantitative capillary electrophoresis sodium dodecyl sulfate method for quality control and stability monitoring of monoclonal antibodies. Anal Chem 78:6583–6594
26. Schmalzing D, Nashabeh W (1997) Capillary electrophoresis based immunoassays: a critical review. Electrophoresis 18:2184–2193
27. Jing P, Kaneta T, Imasaka T (2002) Band broadening caused by the multiple labeling of proteins in micellar electrokinetic chromatography with diode laser-induced fluorescence detection. J Chromatogr A 959:281–287
28. Rodat-Boutonnet A, Naccache P, Morin A et al (2012) A comparative study of LED-induced fluorescence and laser-induced fluorescence in SDS-CGE: application to the analysis of antibodies. Electrophoresis 33:1709–1714

Chapter 2

Discrimination of Glycoproteins from Unglycosylated Proteins in Capillary Electrophoresis: Two-Color LIF Detection Coupled with Post-column Derivatization

Takashi Kaneta

Abstract

Glycosylation is one of the most important posttranslational modifications (PTMs) which lead to the functionalization of proteins. Here, we describe one method for discriminating glycosylated proteins from unglycosylated ones in their mixture sample by capillary electrophoretic separation and two-color laser-induced fluorescence detection coupled with post-column derivatization. Two lasers emitting at 450 and 532 nm permit the detection of amino groups of proteins derivatized by naphthalene-2,3-dicarboxaldehyde and a fluorescently labeled lectin, tetramethylrhodamine-labeled concanavalin A (Rh-Con A), respectively. When a protein mixture react with Rh-Con A, the glycoproteins bound with Rh-Con A exhibit signals at the same migration time in two electropherograms obtained by 450- and 532-nm lasers whereas unbound proteins show a signal only in the electropherogram of the 450-nm laser. So, when one protein is glycosylated it is detected at the same migration time in the electropherograms obtained by two lasers.

Key words Capillary electrophoresis, Glycoprotein, Postcolumn derivatization, Two-color laser-induced fluorescence

1 Introduction

Proteins play many roles in biological systems such as enzymatic reactions, immunological reactions, and maintenance of structure in cell membranes and organs. Proteins are synthesized in biological cells through translation of genes, and then are subject to post-translational modifications (PTMs) such as glycosylation, phosphorylation, and methylation, which lead to the functionalization of proteins in biological systems. Among these PTMs, glycosylation of proteins has key roles in cellular recognition, protein folding, and protein trafficking [1] so that discrimination of glycoproteins from unglycosylated proteins is an important issue in the study of biological systems.

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Several analytical methods have been developed for glycoproteins, glycans, and glycoforms based on chromatographic or electrophoretic separations coupled with mass spectrometry (MS) [2–5]. However, intact proteins are, in general, digested by enzymes since they are too large to be measured directly by MS. Conversely, capillary electrophoresis (CE) has several advantages in protein analysis because of its high resolution of large proteins, rapid separation, and low consumption of samples. Therefore, CE showed excellent performance in the analyses of glycans [6], glycoforms [7], and glycoproteins [8].

In protein analyses, laser-induced fluorescence (LIF) is a high sensitive method that permits the detection of single molecules [9] and the analysis of single cells [10]. However, one of the difficulties in coupling LIF with CE is the need to label proteins with a fluorescent dye before separation. Conversely, post-column [11] and on-column [12] derivatizations are advantageous since native proteins can be injected directly into a capillary with no labeling. Previously, we employed the post-column derivatization for proteins separated by capillary sieving electrophoresis, which permitted direct injection of denatured proteins and their LIF detection [13, 14].

We also developed a novel LIF method to discriminate glycosylated proteins in a protein mixture which contained both glycosylated and unglycosylated proteins [15]. The method consists of CE separation followed by LIF detection with two visible lasers coupled with post-column derivatization. A glycoprotein, thyroglobulin, was clearly discriminated from bovine serum albumin which is not glycosylated using a lectin probe, concanavalin A labeled with tetramethylrhodamine (Rh-Con A).

2 Materials

Prepare all solutions using analytical grade reagents and ultrapure water (*see* **Note 1**). Store all protein solutions and labeling reagents in a refrigerator at 4 °C. Store all buffer solutions at room temperature. Filter all solutions with 0.2-μm membrane filter before the use (*see* **Note 2**).

Use a CE system equipped with a laser-induced fluorescence (LIF) detector and a post-column reactor using sheath flow, in which a separation capillary was inserted into a large bore capillary to react the analytes with the labeling reagent at the outlet of the separation capillary (Fig. 1) [16]. Regulate the collection of the fluorescence signals and the switching of the beam stoppers synchronously by a LabView program (National Instruments, CA, USA) using a personal computer equipped with an A/D converter (NI 9215, National Instruments, CA, USA).

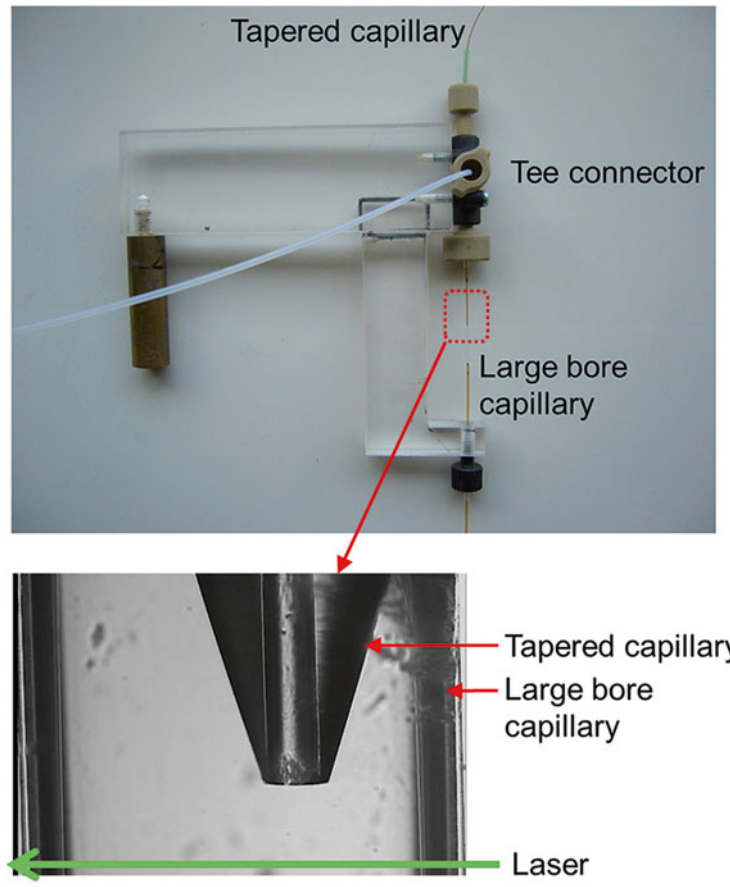

Fig. 1 Photos of the capillary holder (*upper*) and the tip of the tapered capillary inserted into the large bore capillary (*lower*)

2.1 Preparation of Solutions

1. Running buffer for capillary electrophoresis: 50 mM sodium borate buffer. Weigh 4.767 g of sodium tetraborate decahydrate (Borax, MW = 381.37) in a 200 mL beaker. Add about 100 mL of water into the beaker and dissolve the powder in an ultrasonic bath. Transfer the solution to a 250-mL volumetric flask and make up to 250 mL with water.
2. Derivatization solution: 100 mM sodium borate buffer. Weigh 3.814 g of sodium tetraborate decahydrate (Borax, MW = 381.37) in a 100 mL beaker. Add about 50 mL of water into the beaker and dissolve the powder in an ultrasonic bath. Transfer the solution to a 100-mL volumetric flask and make up to 100 mL with water.
3. 5 mM Naphthalene-2,3-dicarboxaldehyde (NDA) solution: 5 mM NDA. Weigh 0.0184 g of NDA in a 20 mL vial and add 20 mL of methanol to dissolve the powder (*see* **Note 3**).

4. 80 mM 2-Mercaptoethanol (2-ME) solution: 80 mM 2-ME. Weigh 0.125 g of 2-ME in a 20 mL vial and add 20 mL of methanol (*see* **Note 4**). Store the solutions of NDA and 2-ME in a refrigerator.
5. Derivatization solution. Mix 7 mL of 100 mM sodium borate buffer, 2 mL of 5 mM NDA, and 1 mL of 80 mM 2-ME, resulting in a solution containing 1 mM NDA, 8 mM 2-ME, 70 mM borate, and 30% methanol.
6. Protein solutions (*see* **Note 5**): 4.9×10^{-5} M Thyroglobulin solution. Weigh 0.0032 g of thyroglobulin in a 1-mL centrifugal tube and add 100 μL of the running buffer. 4.9×10^{-4} M Albumin solution. Weigh 0.0032 g of albumin in a 1-mL centrifugal tube and add 100 μL of the running buffer (50 mM borate) (*see* **Note 6**).
7. Metal solutions: 20 mM $MnCl_2$ solution. Weigh 0.0792 g of Manganese (II) chloride tetrahydrate in a 10 mL beaker. Add about 10 mL of water into the beaker and dissolve the powder in an ultrasonic bath. Transfer the solution to a 20-mL volumetric flask and make up to 20 mL with water. 20 mM $CaCl_2$ solution. Weigh 0.0588 g of calcium chloride dihydrate in a 10-mL beaker. Add about 10 mL of water into the beaker and dissolve the powder in an ultrasonic bath. Transfer the solution to a 20-mL volumetric flask and make up to 20 mL with water.
8. Rh-Con A: Obtain rhodamine concanavalin A from Vector Laboratories (catalog No.: RL-1002, CA, USA), store in a refrigerator at 4 °C, and use without any pretreatment. The commercially available Rh-Con A solution contains 25 mg of protein at the concentration of 5.0 mg/mL as active conjugate, 10 mM HEPES buffer (pH 7.5), 0.15 M NaCl, 0.1 mM Ca^{2+}, 0.01 mM Mn^{2+}, and 0.08% sodium azide (*see* **Note 7**).
9. 2.5×10^{-5} M sodium fluorescein: Weigh 0.004 g of sodium fluorescein in a 50 mL beaker. Add about 30 mL of water into the beaker and dissolve the powder in an ultrasonic bath. Transfer the solution to a 100-mL volumetric flask and make up to 100 mL with water to prepare a stock solution of 0.1 mM sodium fluorescein. Take 2.5 mL of 0.1 mM sodium fluorescein into a 10-mL volumetric flask and make up to 10 mL with water.

2.2 Sample Preparation

1. Sample of thyroglobulin: Take 10 μL of 4.9×10^{-5} M thyroglobulin, 25 μL of 2.5×10^{-5} M sodium fluorescein (internal standard), and 65 μL of 50 mM sodium borate (running buffer) into a 1-mL centrifugal tube.
2. Sample of Rh-Con A: Take 10 μL of Rh-Con A, 2.5 μL of 20 mM $MnCl_2$ solution, 2.5 μL of 20 mM $CaCl_2$ solution (*see* **Note 8**), 25 μL of 2.5×10^{-5} M sodium fluorescein, and 60 μL of 50 mM sodium borate (running buffer) into a 1-mL centrifugal tube.

3. Sample of thyroglobulin containing Rh-Con A: Take 10 μL of 4.9×10^{-5} M thyroglobulin, 10 μL of Rh-Con A, 2.5 μL of 20 mM $MnCl_2$ solution, 2.5 μL of 20 mM $CaCl_2$ solution, 25 μL of 2.5×10^{-5} M sodium fluorescein (internal standard), and 50 μL of 50 mM sodium borate (running buffer) into a 1-mL centrifugal tube.
4. Sample of albumin: Take 10 μL of 4.9×10^{-4} M albumin (*see* **Note 9**), 25 μL of 2.5×10^{-5} M sodium fluorescein (internal standard), and 65 μL of 50 mM sodium borate (running buffer) into a 1-mL centrifugal tube.
5. Sample of albumin containing Rh-Con A: Take 10 μL of 4.9×10^{-4} M albumin, 10 μL of Rh-Con A, 2.5 μL of 20 mM $MnCl_2$ solution, 2.5 μL of 20 mM $CaCl_2$ solution, 25 μL of 2.5×10^{-5} M sodium fluorescein, and 50 μL of 50 mM sodium borate (running buffer) into a 1-mL centrifugal tube.
6. Mixture sample of thyroglobulin and albumin containing Rh-Con A: Take 10 μL of 4.9×10^{-5} M thyroglobulin, 10 μL of 4.9×10^{-4} M albumin, 10 μL of Rh-Con A, 2.5 μL of 20 mM $MnCl_2$, 2.5 μL of 20 mM $CaCl_2$, 25 μL of 2.5×10^{-5} M sodium fluorescein, and 40 μL of 50 mM sodium borate (running buffer) into a 1-mL centrifugal tube.

2.3 Post-column Reactor

1. Fill a tapered capillary (50 μm i.d., 360 μm o.d., and 40 cm length, PicoTip EMITTER, TT360-50-50-CE-5, coating; P200P, NEW OBJECTIVE, MA, USA) with 0.1 M NaOH by a 100-μL microsyringe connected with the inlet of the capillary using a piece of polytetrafluoroethylene tube and activate the inner surface of the capillary overnight (*see* **Note 10**).
2. Connect the tapered capillary with a tee connector fixed on a home-made holder (Fig. 1).
3. Connect a large bore capillary (530 μm i.d., 660 μm o.d., and 13 cm length, GL Science, Tokyo, Japan) with the tee connector so as to insert the tip of the tapered capillary into the large bore capillary (Fig. 1) (*see* **Note 11**).

2.4 LIF Detector

1. Align two lasers emitting at 450 nm (20 mW Z20M18H-F-450-pe, Z-LASER, Germany) and 532 nm (40 mW, Z40M18B-F-532-pz, Z-LASER, Germany), a dichroic filter (XF2077, Omega Optical, Inc., VT, USA), aluminum mirrors, a plane-convex lens (synthesized quartz, $\phi = 10$ mm, focal distance=50 mm) for focusing the laser beams, and a detection box consisting of a photomultiplier tube (Model R3896, Hamamatsu, Shizuoka, Japan), a pinhole ($\phi = 0.5$ mm), and a microscope objective (Olympus, LWD LSPlan 50×/N. A., 0.60) for collecting the fluorescence signal (Fig. 2).
2. Overlap the two laser beams by adjusting the dichroic filter, mirror 1, and mirror 2 (*see* **Note 12**) and locate the two beam

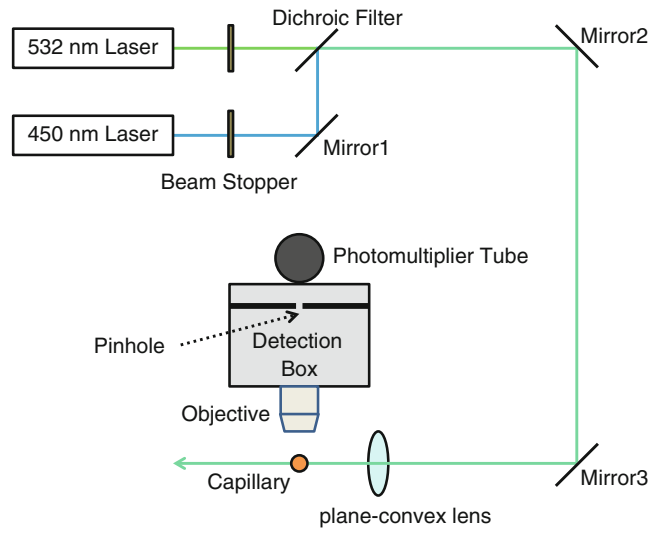

Fig. 2 Schematic illustration of optics alignment

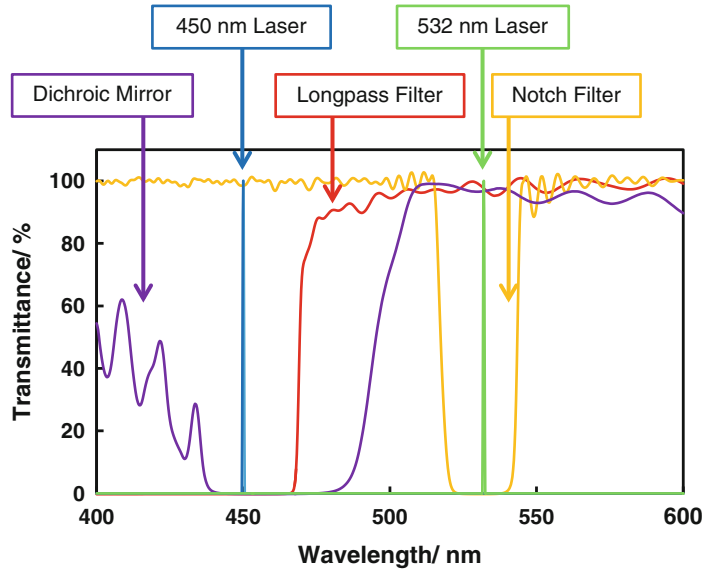

Fig. 3 Transmission curves of the optical filters. The emission wavelengths of the lasers are indicated by lines (450 and 532 nm)

stoppers (F116-1, SURUGA SEIKI, Shizuoka, Japan) controlled by a shutter controller (F77-7, SURUGA SEIKI, Shizuoka, Japan) between the lasers and the dichroic filter (Fig. 2).

3. Place a long-pass filter (HQ470LP, Chroma Technology Corporation, VT, USA), and notch filters (NF01-532U-25, Semrock, NY, USA and 532 nm Rugate Notch Filter, Edmund Optics Inc., NJ, USA) (Fig. 3) between the microscope objective and the photomultiplier tube in the detection box.

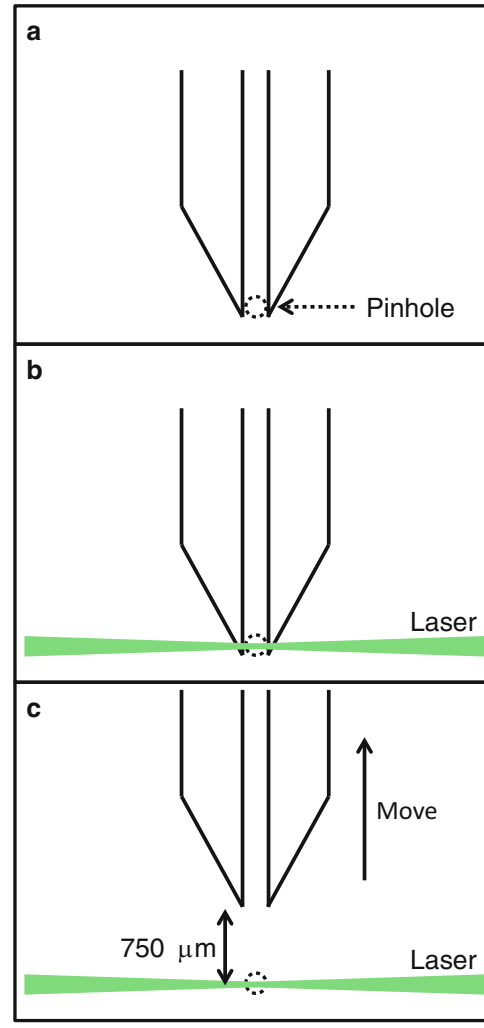

Fig. 4 Alignment of the capillary and the laser beams in the post-column fluorescence detection

4. Adjust the capillary so as that the center of the tapered capillary overlaps with the image of the pinhole placed in front of the photomultiplier tube in the detection box (Fig. 4a).
5. Focus the laser beams onto the tapered capillary, and then move the laser beams so as to overlap with the pinhole (Fig. 4b) by moving the plane-convex lens.
6. Move the capillary to adjust the distance between the tip of the capillary and the laser beams to 750 μm (Fig. 4c) (*see* **Note 13**).
7. Observe change in the fluorescent signal by injecting a fluorophore solution such as fluorescein and rhodamine B with a microsyringe to confirm whether alignment of the detection system is correct or not.

3 Methods

1. Condition the separation capillary (tapered capillary) by rinsing with 0.1 M NaOH for 5 min, deionized water for 5 min, and the running buffer for 5 min, sequentially.
2. Condition the reaction capillary (large bore capillary) by rinsing with water and the running buffer sequentially.
3. Fill the separation capillary and the reaction capillary with the running buffer and the derivatization solution, respectively (*see* **Note 14**).
4. Inject a sample solution (one of them described in Subheading 2.2) hydrodynamically for 10 s into the capillary from the sample vial raised 10 cm above the outlet vial (*see* **Note 15**).
5. Apply a constant potential of +10 kV to the inlet side of the separation capillary by a high-voltage power supply (HCZE-30PN0.25, Matsusada Precision, Shiga, Japan), and flow the derivatization solution into the reaction capillary at a flow rate of 0.2 μl/min during the separation (*see* **Note 16**).
6. Save the data as a text file, and then open the text using a spreadsheet software such as Excel to show two electropherograms (Fig. 5 (the results for (A) thyroglobulin, (B) Rh-Con A, and (C) their mixture), Fig. 6 (the results for (A) albumin, (B) Rh-Con A, and (C) their mixture), Fig. 7 (the results for a mixture of thyroglobulin, albumin, and Rh-Con A) (*see* **Note 17**).
7. Fill the separation capillary with 0.1 M NaOH to keep the surface of the capillary activated after the experiments.
8. Flush the reaction capillary with 0.1 M NaOH and fill it with water after the experiment (*see* **Note 18**).

4 Notes

1. Filter pure water before the use for preparation of solutions. A disposable syringe filter was usually employed for rapid filtration.
2. The filtration helps removing particulate matters in solutions since they appear as spike noises in the electropherogram due to scattering of laser light.
3. Store the NDA solution in a refrigerator within 1 week and prepare a fresh solution every week.
4. Wear gloves and weigh in a fume hood for the preparation of the solution since 2-ME is toxic and volatile.

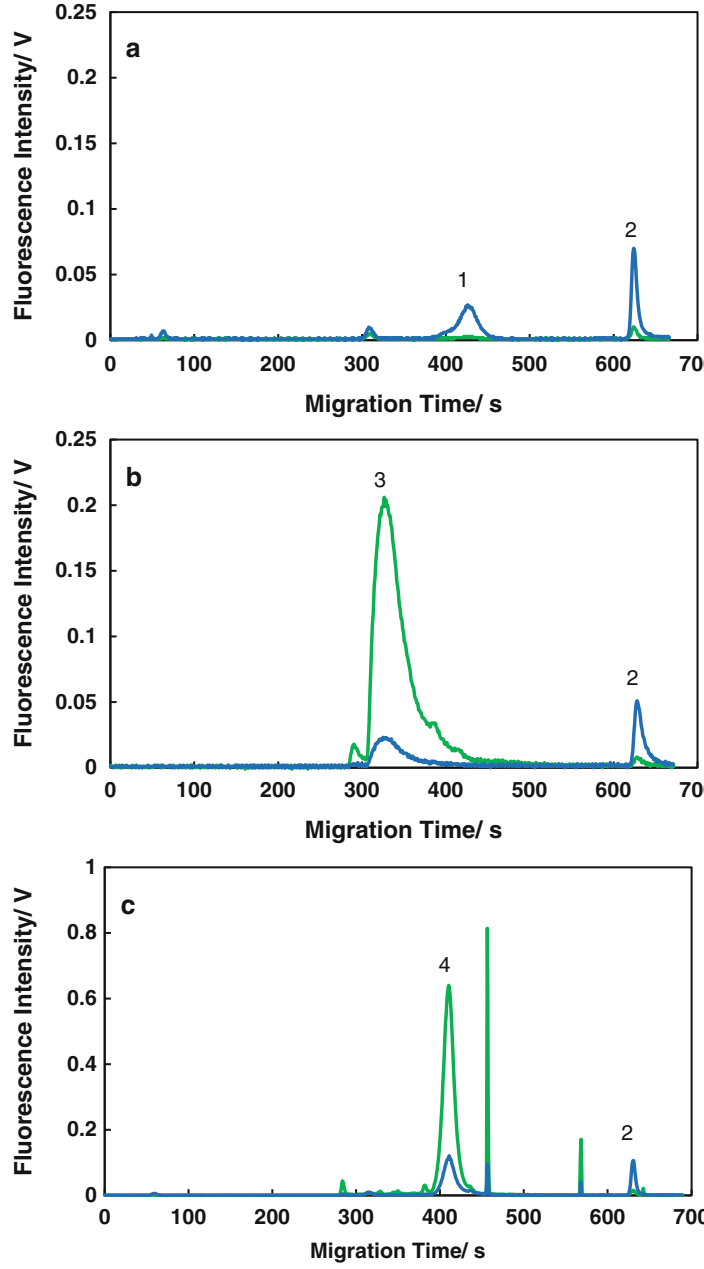

Fig. 5 Electropherograms of thyroglobulin, Rh-Con A, and their mixture. (**a**) 4.9 μM thyroglobulin (sample 1), (**b**) 4.9 μM Rh-Con A (sample 2), (**c**) a mixture of 4.9 μM thyroglobulin and 4.9 μM Rh-Con A (sample 3). (1) thyroglobulin, (2) fluorescein, (3) Rh-ConA, (4) complex between thyroglobulin and Rh-Con A. *Blue lines* and *green lines* are obtained by 450-nm excitation and 532-nm excitation, respectively. Reproduced from [15] with permission from John Wiley and Sons

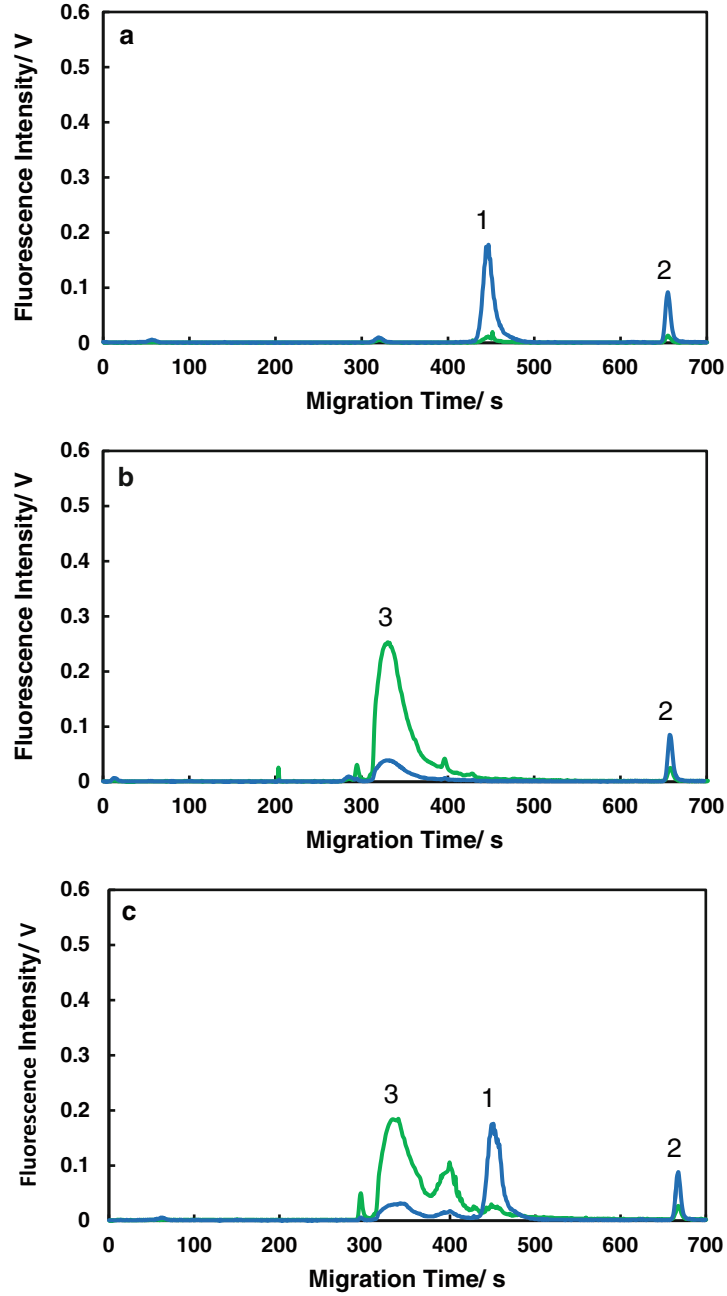

Fig. 6 Electropherograms of albumin, Rh-Con A and a mixture of albumin and Rh-Con A. (**a**) 49 μM albumin (sample 4), (**b**) 4.9 μM Rh-Con A (sample 2), (**c**) 49 μM albumin with 4.9 μM Rh-Con A (sample 5). (1) Albumin, (2) fluorescein, (3) Rh-Con A. *Blue lines* and *green lines* are obtained by 450-nm excitation and 532-nm excitation, respectively. Reproduced from [15] with permission from John Wiley and Sons

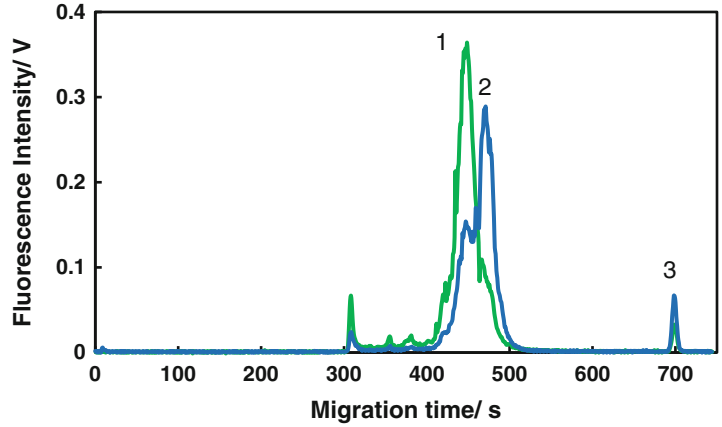

Fig. 7 Electropherogram of a mixture of thyroglobulin, albumin, and Rh-Con A (sample 6). (1) Complex between thyroglobulin and Rh-Con A, (2) albumin, (3) fluorescein. *Blue lines* and *green lines* are obtained by 450-nm excitation and 532-nm excitation, respectively. Reproduced from [15] with permission from John Wiley and Sons

5. Employ molecular masses of 660 and 66 kDa for thyroglobulin and albumin, respectively, to calculate the concentrations of the protein solutions.
6. Prepare fresh samples when the peaks of proteins are distorted.
7. The concentration of the active concanavalin A was calculated to be 4.9×10^{-5} M, using molecular mass of 102,000 for concanavalin A. The number of conjugated fluorophore to one concanavalin A molecule given by the manufacture was 3.5, that is, the product was a mixture of multiply labeled Rh-Con A molecules.
8. The concentrations of $CaCl_2$ and $MnCl_2$ must be kept at 0.5 mM in order to obtain a sharp peak for Rh-Con A.
9. Note that the concentration of albumin is 10 times higher than that of thyroglobulin due to the poor sensitivity of albumin-NDA derivative.
10. Suppress adsorption of negatively charged proteins on the capillary surface by activation. The surface of a fused-silica capillary was charged negatively by flushing 0.1 M NaOH.
11. Insert the tapered capillary into the large bore capillary carefully, connect the large bore capillary with the tee connector, and then fix the large bore capillary on the holder so as not to damage the tip of the tapered capillary.
12. Adjust the height of two laser beams at 12 cm which is the same height as that of the detector, adjust the angles of the laser beams to be parallel to the plain face of a laboratory table by moving the lasers, and then overlap the laser beams using the dichroic filter and mirrors.

13. Fix the capillary holder on a stage equipped with three-dimensional micrometer heads. Adjust the distance between the laser beam and the capillary tip by the micrometer head for *Z*-axis.
14. Bubbles must be completely removed from the tapered capillary and the large bore capillary. To remove bubbles, both the buffer solution and the derivatization solution were flushed fast.
15. Keep the length between the liquid levels of the outlet reservoir and the sample reservoir 10 cm.
16. Monitor the electric current to confirm the application of an electric field. The electric current is, in general, constant at 5 μA under the present conditions.
17. Check alignment of the laser, the capillary, and the pin-hole when no signal is obtained. The reason of no signal is, in most cases, attributed to wrong alignment of the detection system. Another reason is due to no injection of the sample.
18. Keep the separation capillary activated after the experiment.

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References

1. Tian Y, Zhang H (2010) Glycoproteomics and clinical applications. Proteomics Clin Appl 4:124–132
2. Carr SA, Huddleston MJ, Bean MF (1993) Selective identification and differentiation of *N*- and *O*-linked oligosaccharides in glycoproteins by liquid chromatography–mass spectrometry. Protein Sci 2:183–196
3. Balaguer E, Neusüss C (2006) Glycoprotein characterization combining intact protein and glycan analysis by capillary electrophoresis–electrospray ionization-mass spectrometry. Anal Chem 78:5384–5393
4. Balaguer E, Demelbauer U, Pelzing M, Sanz-Nebot V, Barbosa J, Neusüß C (2006) Glycoform characterization of erythropoietin combining glycan and intact protein analysis by capillary electrophoresis–electrospray–time-of-flight mass spectrometry. Electrophoresis 27:2638–2650
5. Nakano M, Higo D, Arai E, Nakagawa T, Kakehi K, Taniguchi N, Kondo A (2009) Capillary electrophoresis–electrospray ionization mass spectrometry for rapid and sensitive N-glycan analysis of glycoproteins as 9-fluorenylmethyl derivatives. Glycobiology 19:135–143
6. Lamari F, Militsopoulou M, Gioldassi X, Karamanos NK (2001) Capillary electrophoresis: a superior miniaturized tool for analysis of the mono-, di-, and oligosaccharide constituents of glycan moieties in proteoglycans. Fresenius J Anal Chem 371:157–167
7. Kakehi K, Kinoshita M, Kawakami D, Tanaka J, Sei K, Endo K, Oda Y, Iwaki M, Masuko T (2001) Capillary electrophoresis of sialic acid-containing glycoprotein. Effect of the heterogeneity of carbohydrate chains on glycoform separation using an α1-acid glycoprotein as a model. Anal Chem 73:2640–2647

8. Werner WE, Demorest DM, Wiktorowicz JE (1993) Automated Ferguson analysis of glycoproteins by capillary electrophoresis using a replaceable sieving matrix. Electrophoresis 14:759–763
9. Weiss S (1999) Fluorescence spectroscopy of single biomolecules. Science 283:1676–1683
10. Zhang ZR, Krylov S, Arriaga EA, Polakowski R, Dovichi NJ (2000) One-dimensional protein analysis of an HT29 human colon adenocarcinoma cell. Anal Chem 72:318–322
11. Gilman SD, Pietron JJ, Ewing AG (1994) Post-column derivatization in narrow-bore capillaries for the analysis of amino acids and proteins by capillary electrophoresis with fluorescence detection. J Microcol Sep 6:373–384
12. Gilman SD, Ewing AG (1995) Analysis of single cells by capillary electrophoresis with on-column derivatization and laser-induced fluorescence detection. Anal Chem 67:58–64
13. Kaneta T, Yamamoto D, Imasaka T (2009) Postcolumn derivatization of proteins in capillary sieving electrophoresis/laser-induced fluorescence detection. Electrophoresis 30:3780–3785
14. Kaneta T, Ogura T, Imasaka T (2011) Analysis of proteins in biological samples by capillary sieving electrophoresis with postcolumn derivatization/laser-induced fluorescence detection. Electrophoresis 32:1061–1067
15. Tabara A, Kaneta T (2013) Discrimination of glycoproteins via two-color laser-induced fluorescence detection coupled with postcolumn derivatization in capillary electrophoresis. Electrophoresis 34:2316–2322
16. Rose DJ Jr, Jorgenson JW (1988) Post-capillary fluorescence detection in capillary zone electrophoresis using *o*-phthaldialdehyde. J Chromatogr 447:117–131

Chapter 3

Capillary Zone Electrophoresis–Mass Spectrometry of Intact Proteins

Elena Domínguez-Vega, Rob Haselberg, and Govert W. Somsen

Abstract

Capillary electrophoresis (CE) coupled with mass spectrometry (MS) has proven to be a powerful analytical tool for the characterization of intact proteins. It combines the high separation efficiency, short analysis time, and versatility of CE with the mass selectivity and sensitivity offered by MS detection. This chapter focuses on important practical considerations when applying CE-MS for the analysis of intact proteins. Technological aspects with respect to the use of CE-MS interfaces and application of noncovalent capillary coatings preventing protein adsorption are treated. Critical factors for successful protein analysis are discussed and four typical CE-MS systems are described demonstrating the characterization of different types of intact proteins by CE-MS. These methodologies comprise the use of sheath-liquid and sheathless CE-MS interfaces, and various types of noncovalent capillary coatings allowing efficient and reproducible protein separations. The discussion includes the analysis of lysozyme-drug conjugates and the therapeutic proteins human growth hormone, human interferon-β-1a, and human erythropoietin.

Key words Capillary electrophoresis, Electrospray ionization, Mass spectrometry, sheath-liquid interface, Sheathless interface, Proteins, Biopharmaceuticals, (Non-)covalent coatings

1 Introduction

Advances in pharmaceutical biotechnology and protein chemistry have stimulated a growing interest in improved analytical tools for the study and characterization of intact proteins. Function and activity of proteins strongly relies on molecular structure, including the presence (or absence) of specific posttranslational modifications. For example, pharmaceutical proteins may undergo various modifications during production, downstream processing, and storage. These may dramatically influence bioactivity, efficacy, and toxicity (e.g. immunogenicity), thereby compromising their therapeutic properties. Quality control of biopharmaceuticals is an essential—but challenging—task, requiring analytical techniques allowing characterization of intact proteins and their variants.

Due to its intrinsic properties, capillary electrophoresis (CE) has emerged as a powerful technique for the separation of intact

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proteins [1]. CE is carried out in an open narrow tube—usually a fused-silica capillary—filled with a background electrolyte solution. CE analyses can be performed in absence of stationary phase under mild, interaction-free conditions, without the need for organic solvents or very high salt concentrations. This allows the study of proteins without inducing unwanted conformational changes, binding or degradation during analysis. CE separations are based on differences in molecular charge-to-size ratio. Therefore, subtle structural differences among proteins, such as caused by (posttranslational) modifications (e.g. glycosylation, deamidation, or phosphorylation) can be revealed by CE.

Mass spectrometry (MS) has developed into one of the most popular and useful detection techniques in separation science because of its sensitivity and selectivity. Furthermore, MS detection with high mass accuracy and resolution, such as provided by time-of-flight (TOF) instruments, can considerably enhance the utility of CE by providing information about the identity of the separated compounds. Therefore, coupling CE to MS creates a powerful analytical tool for the characterization of intact proteins [2–4]. Electrospray ionization (ESI) is the dominant ionization technique used in CE-MS of proteins because of its effectiveness for large biomolecules and simplicity of use. ESI produces multiple-charged protein ions in the gas phase. As a result, a so-called protein charge envelope comprising ions with mass-to-charge ratios of typically 500–3000 is obtained. Protein molecular masses can be determined by deconvolution of the ESI mass spectrum.

For coupling CE with ESI-MS, different specific aspects should be taken into account. In CE-MS, volatile buffers of relatively low conductivity are required to keep CE currents below 50 μA to obtain stable electrospray and to avoid arcing between the spray chamber and the mass spectrometer. In general, background electrolytes (BGEs) composed of formic acid or acetic acid, or of ammonium or (bi)carbonate are employed. CE-MS requires closure of both the CE and ESI electrical circuit. Furthermore, as CE effluent flow rates are very low (nL/min), direct coupling with conventional ESI sources designed to work with flows in μL/min range is not possible. In order to facilitate CE-MS, a dedicated interface is required to achieve proper voltage and flow rate handling. Several interfaces have been developed over the last years [5, 6], but only few of them are commercially available.

The most common interface employed for CE-MS coupling is the so-called sheath-liquid interface commercialized by Agilent Technologies (Fig. 1a). In the sheath-liquid interface, the CE capillary is surrounded by a stainless steel tube in a coaxial arrangement. A sheath liquid is led through this tube, which merges with the CE effluent at the capillary outlet allowing closure of the CE electrical circuit. The tip of the stainless steel tube serves also as electrospray needle. Typical voltages between the sprayer and the

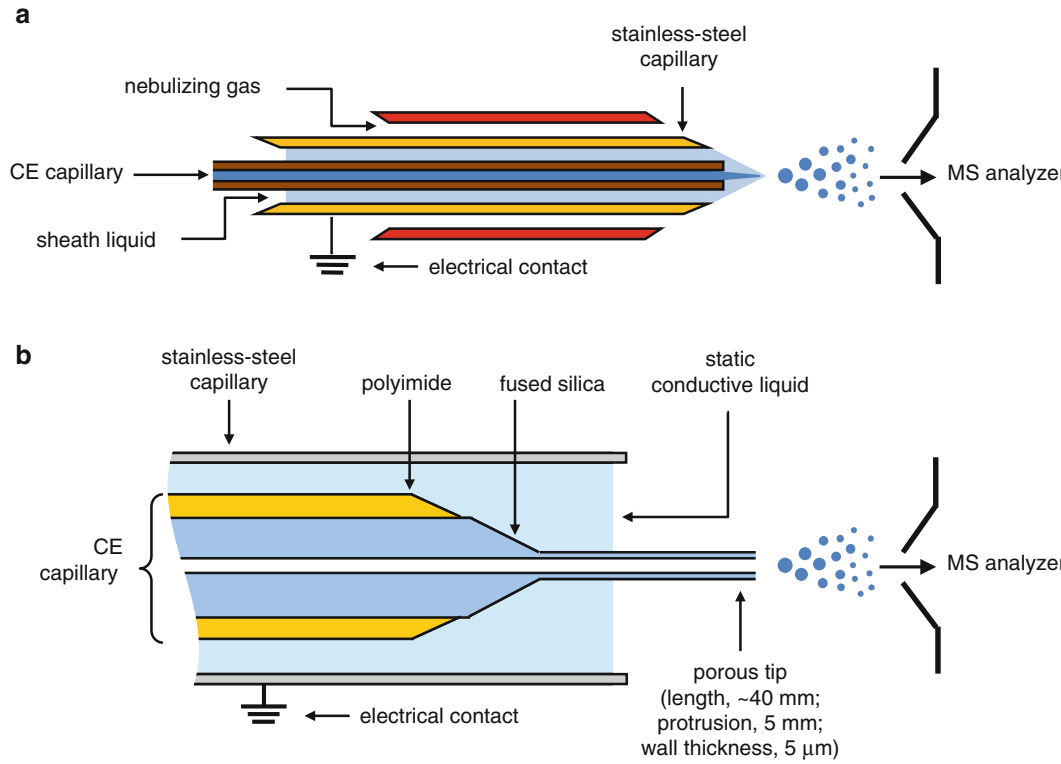


Fig. 1 Schematic representation of a sheath-liquid (**a**) and a sheathless (**b**) CE-MS interface

entrance of the mass spectrometer are in the range of 2–5 kV. Proteins are generally determined in positive ion mode as they are effectively protonated to form positively charged ions during ESI. A third coaxial tube delivers a gas flow that aids nebulization of the CE effluent into the ion source. The nebulizer gas can produce a small suction of the CE effluent. This effect could cause band broadening and decrease in separation efficiency and resolution and, therefore, should be evaluated. The sheath-liquid composition is essential [7, 8], because it importantly determines the protein signal intensity as well as the shape and position of the protein's charge state distribution. Common sheath liquids contain an organic solvent, water, and a volatile acid. The sheath liquid flow rate should have a flow sufficient to allow aerosol formation in the ESI source. However, the use of the sheath liquid produces dilution of the capillary effluent and as a result detection sensitivity may be compromised. Therefore, too large flows should be avoided to prevent too much dilution of the capillary effluent. Flows between 2 and 5 μL/min are recommended. Overall, the sheath-liquid interface is robust and provides good flexibility for CE-MS coupling.

An alternative to sheath-flow interfacing for CE-MS coupling are the so-called sheathless interfaces. In this type of interface the

CE electrical circuit is closed directly using the BGE just before or after it leaves the capillary, eliminating the need for sheath-liquid. This can be achieved by using different strategies as, e.g. connecting a metal-coated sprayer tip to the CE, using metal coating to the end of the tapered separation capillary, or introducing a microelectrode into the end of the CE capillary. However, most of these designs are lab-made and not commercially available [6, 9]. Recently, a novel sheathless interface to couple CE with MS has been designed which is now commercially available. The interface was developed in the laboratories of Beckman Coulter (now Sciex Separations), based on the concept introduced by Moini [10]. In this design (Fig. 1b), 3–4 cm of the terminating end of the fused-silica separation capillary is etched with hydrofluoric acid, producing a ~5-μm thick porous capillary wall, which is conductive when in contact with an electrolyte. The electrical contact for both the CE and ESI is achieved by letting the porous capillary outlet protrude from a stainless steel ESI needle filled with static conductive liquid allowing electrospray formation at the capillary tip. In addition, as effluent volume flow rates are low, the initial droplets formed during the electrospray process are small, leading to more efficient ionization (i.e. nanospray) [11]. Therefore, the nebulizer gas is not necessary and possible problems derived from suction effects are avoided. This approach also allows the ESI spray tip to be positioned closer to the MS inlet and, thereby, ion sampling efficiencies are improved [11, 12]. The ESI voltage necessary to ensure proper electrospray formation is dependent on various factors as, e.g. the position of the capillary tip to the MS source, and can dramatically affect the sensitivity of the analysis. Typical ESI voltages in sheathless CE-MS are in the range of 1–2 kV. As sheath liquid is not necessary for the closure of the electrical circuit, the CE effluent is not diluted and improved sensitivity may be obtained. This novel interface has been evaluated for the analysis of intact proteins in different occasions [13, 14]. Limits of detection (LODs) between 0.5 and 1.3 nM were obtained for intact proteins with the sheathless CE-MS system using a nanoESI source, which was 50- to 140-fold better than sheath-liquid interfacing using the same capillary [13]. The gain in sensitivity was the result of both reduced noise levels and increased analyte responses as obtained with sheathless CE-MS.

Because proteins have relatively low diffusion coefficients theoretically in CE-MS high plate numbers can be obtained. However, a critical practical limitation encountered is the tendency of intact proteins to adsorb to fused silica, causing serious peak tailing and migration time shifts [15]. The most common approach in CE to prevent protein adsorption is to coat the inner capillary wall with surface coating agents. Various chemical coatings have been successfully employed to prevent adsorption of proteins to the capillary wall, including dynamic (coating agent

is present in the BGE and is removed during rinsing steps) and static coatings (capillary is coated before use and the coating remains on the capillary surface during rinsing and separation steps) [9, 16]. In CE-MS the coating should not interfere with MS detection. Coating material entering the mass spectrometer could lead on ionization suppression, contamination of the mass spectrometer, and/or background noise. Therefore, in CE-MS of proteins stable static coatings are used. An elegant and simple way to create effective and stable static coatings is by applying charged polymers to the fused-silica surface [17]. The usefulness of noncovalent polyelectrolyte coatings as polyethyleneimine silane (PEI), polybrene-poly(vinylsulfonic acid) (PB-PVS), or polybrene-dextran sulfate-polybrene (PB-DS-PB) for the CE-MS analysis of intact proteins has been amply demonstrated [13, 16, 18–21]. Based on the principle of electric repulsion, the negatively charged PB-PVS coating can be used in combination with a BGE of medium or high pH for efficient analysis of acidic proteins. In a similar fashion, positively charged coatings (PB-DS-PB or PEI) are applied in combination with low-pH BGEs. These capillary coatings provide highly reproducible and efficient protein separations. Typical migration time RSD values are within 1.5 % ($n = 40$), whereas plate numbers for intact proteins are generally around 100,000 [21, 22]. Because of the permanent charge of the coating polymers, a constant and pH-independent electro-osmotic flow (EOF) is obtained. However, the significant EOF also may decrease the effective separation window and resolution which can be limiting when complex samples or heterogeneous proteins have to be analyzed. Polyacrylamide coatings reduce the EOF velocity to virtually zero allowing to achieve enhanced CE resolution of proteins at the cost of prolonged analysis times [23, 24].

In this chapter, four CE-MS methodologies are described in order to offer the reader a practical overview of the available strategies for the analysis of intact proteins by CE-MS. These methodologies comprise the use of sheath-liquid and sheathless interfaces, as well as different types of capillary coatings. The first two methodologies encompass the use of sheath-liquid CE-MS for the analysis of an acidic protein (recombinant human growth hormone, rhGH) and basic proteins (recombinant human interferon-β-1a, rhIFN-β) using negatively and positively charged coated capillaries, respectively. The other two methods pay attention to the use of a sheathless interface for the analysis of intact proteins. First, the use of a positively charged porous-tip capillary for the analysis of lysozyme (LZM)-drug conjugates is described. In the last example, the use of a neutral porous-tip capillary in combination with an acidic BGE is outlined as a high-resolution method for the separation of recombinant human erythropoietin (rhEPO) glycoforms.

2 Materials

2.1 Instrumentation

1. In the studies described below, a Beckman Coulter MDQ CE instrument was coupled to a Bruker Daltonics micrOTOF mass spectrometer using a CE-MS interface from either Agilent Technologies (Palo Alto, CA, USA) or Beckman Coulter/Sciex Separations (Brea, CA, USA).
2. For sheath-liquid CE-MS a syringe of 2.5 mL (Hamilton, Reno, NV, USA) and a syringe pump from Cole-Parmer (Vernon Hill, IL, USA) were used to supply a constant flow of the sheath liquid.
3. The regular ESI endplate and capillary cap, and low-flow spacer provided by Bruker Daltonics were used in sheath-liquid interfacing. A nanospray end plate and gas diverter were installed to allow nanoESI for the sheathless experiments.
4. Bare fused-silica capillaries with an effective length of 80 cm and an internal diameter of 50 μm were used for the examples described in Subheadings 3.3.1 and 3.3.2.
5. Bare fused-silica and neutral-coated capillaries with an effective length of 100 cm and an internal diameter of 30 μm equipped with a porous tip (3–4 cm of the outlet end) from Beckman Coulter/Sciex Separations were employed for the examples described in Subheadings 3.3.3 and 3.3.4, respectively.

2.2 Chemicals and Solutions

1. Capillary rinsing solutions: 0.1 and 1 M NaOH; 0.1 M HCl.
2. Coating solutions: 10% Polybrene (hexadimethrine bromide, PB; average Mw 15,000) in water; 0.5% dextran sulfate (DS; average MW >500,000) in water; 1% poly(vinylsulfonic acid) sodium salt (PVS; average MW 4000–6000) in water; 20% polyethyleneimine silane (PEI) in anhydrous

MeOH. Prepare PB, PVS, DS, and PEI solutions by dissolving the appropriate amount in deionized water to a final concentration of 10% (w/v), 1% (w/v), 0.5% (w/v), and 20% (v/v) (*see* **Notes 1** and **2**), respectively. Filter the PB, PVS, and DS solutions using a 0.45 μm filter type HA (Millipore, Molsheim, France) prior to use.

3. Sheath liquids: acetonitrile–water–formic acid (75/20/5, v/v/v); acetonitrile–water–acetic acid (75/25/0.5, v/v/v).
4. BGEs: 75 mM ammonium formate (pH 8.5, adjusted with formic acid); 50 mM acetic acid (pH 3.0, adjusted with ammonia); 100 mM acetic acid (pH 3.1) containing 5% (v/v) of isopropanol; 2 M acetic acid (pH 2.1).

2.3 Proteins

1. 1.5 mg/mL recombinant human growth hormone (rhGH, Mw, 22124 Da; pI, 5.0) in water.

2. 0.45 mg/mL recombinant human interferon-β-1a (rhIFN-β, average MW ~22.3 kDa; pI, 8.9) in water.
3. 100 nM erlotinib-lysozyme conjugates in water. Conjugates were prepared by first coupling *N*-(tert-butoxycarbonyl)-l-methionine hydroxysuccinimide ester (BOCmet) to lysine residues of LZM followed by conjugation with erlotinib via a platinum(II)-based linker as described earlier [25, 26].
4. 0.2 mg/mL recombinant human erythropoietin (rhEPO; average Mw ~30 kDa; pI, 4; NeoRecormon 30,000 IU) in water.

3 Methods

3.1 Capillary Conditioning

Before first use the capillaries should be conditioned and coated as indicated below (*see* **Note 3**):

1. Bare fused-silica capillaries (50 μm ID): Flush the capillary with 1 M NaOH (20 psi) for 30 min, followed by 15 min with deionized water (20 psi).
2. Bare fused-silica porous-tip capillaries: Flush the capillary with MeOH (100 psi, 10 min forward and 3 min reverse pressure), followed by deionized water (100 psi, 10 min forward and 3 min reverse pressure), 10 min of 0.1 M NaOH (100 psi, forward pressure), 10 min of 0.1 M HCl (100 psi, forward pressure) and 10 min of deionized water (100 psi, forward pressure) (*see* **Notes 4** and **5**).
3. Neutral porous-tip capillaries: Rinse the capillary with deionized water (50 psi, for 30 min forward and 5 min reverse pressure) followed by the BGE (50 psi, for 10 min forward and 3 min reverse pressure) (*see* **Note 6**).

After this treatment, bare fused-silica capillaries were coated with either a PB-PVS coating or a PB-DS-PB coating in the case of sheath liquid CE-MS, and with a PEI coating in the case of sheathless CE-MS using the procedures described below. Neutral coated capillaries were ready for use after the conditioning step.

3.2 Capillary Coating

3.2.1 PB-PVS Coating

1. Rinse the preconditioned capillaries with 10% PB (30 min, 5 psi), deionized water (10 psi, 10 min), 1% PVS (5 psi, 30 min), and deionized water (10 psi, 10 min).
2. After the final coating step and before CE analysis rinse the capillary with the BGE of choice for 10 min (20 psi) (*see* **Note 7**).

3.2.2 PB-DS-PB Coating

1. Rinse the preconditioned capillaries with 10% PB (5 psi, for 30 min), deionized water (10 psi, 10 min), 0.5% DS (5 psi,

30 min), deionized water (10 psi, 10 min), 10% PB (5 psi, 30 min), and deionized water (10 psi, 10 min).

2. After the final coating step and before CE analysis rinse the capillary with the BGE of choice for 10 min (20 psi) (*see* **Note 7**).

3.2.3 Polyethyleneimine Silane (PEI)

1. Rinse the preconditioned capillary with MeOH (50 psi, 20 min) followed by deionized water (50 psi, 10 min). Subsequently, pump air (50 psi, 5 min) through the capillary. Flush the capillary with the PEI solution for 20 min (50 psi) followed by 10 min of deionized water (50 psi) (*see* **Note 8**).
2. After the final coating step and before CE analysis rinse the capillary with the BGE of choice (50 psi, 10 min).

Overnight, coated capillaries should be filled with deionized water and tips should be immersed in vials containing water. When not in use, store the capillaries filled with deionized water at +2–8 °C. In the particular case of neutral porous-tip capillaries flush with air (50 psi, 10 min) before storage.

3.3 CE-MS Analysis

This example describes a CE-MS methodology using a conventional sheath-liquid interface for the analysis of an acidic protein, recombinant human growth hormone (rhGH). rhGH is a therapeutic protein used in the treatment of retarded growth and dwarfism. In order to obtain reproducible and efficient separations, a negatively charged coated capillary in combination with a slightly basic BGE is selected.

3.3.1 Analysis of rhGH by Sheath-Liquid CE-MS Using PB-PVS Coated Capillaries

1. Condition and coat the capillary as indicated in Subheadings 3.1, **step 1** and 3.2.1.
2. Insert the capillary in the ESI interface letting the capillary tip protrude for approximately 1 mm from the nebulization capillary.
3. Prepare the sheath liquid (acetonitrile–water–formic acid (75/25/5, v/v/v)) and degas it by sonication (*see* **Notes 9** and **10**).
4. Introduce the sheath liquid in a chromatographic syringe and start pumping the sheath liquid using a syringe pump at a flow rate of 4 μL/min.
5. Rinse the capillary with 75 mM ammonium formate at pH 8.5 for 10 min (20 psi).
6. Select the appropriate MS method, set the nebulizer pressure at 0.4 bar and deliver the drying gas at 4 L/min N_2 and 180 °C. Select a scan range of 300–3000 *m/z* (*see* **Note 11**).
7. Turn on the ESI voltage (−4.5 kV).

8. Inject the rhGH sample in the CE instrument by applying pressure to fill 1 % of the capillary (1 psi for 12 s) (*see* **Note 12**).
9. Start the electrophoretic process by applying a voltage of 30 kV using a temperature of 20 °C.
10. Monitor the base-peak electropherogram (BPE) obtained in the range of 1500–3000 *m/z*. Figure 2a shows the BPEs obtained for rhGH and heat exposed rhGH under these conditions.
11. Determine the mass of the observed peaks by deconvolution of the mass spectra. Perform spectral deconvolution as indicated in Subheading 3.4. Mass spectra, molecular masses, and proposed structure of observed peaks for rhGH obtained after deconvolution are shown in Fig. 2b.
12. At the end of the analysis, switch off the electrophoresis voltage and rinse the capillary with BGE (5 min, 20 psi).
13. Inject a new sample (**steps 9–13**) or clean the capillary with water (20 psi, 10 min).

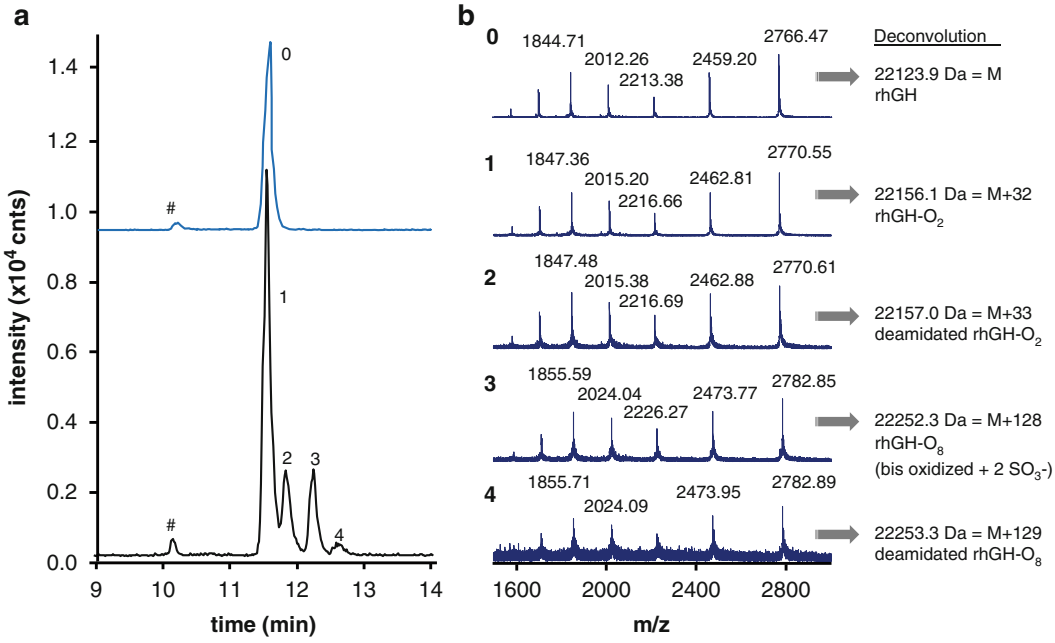


Fig. 2 (**a**) BPEs obtained during CE-TOF-MS of rhGH (1.5 mg/mL, blue trace) and heat-exposed and stored rhGH (1.5 mg/mL, *black* trace) using a PB-PVS coated capillary in combination with a BGE of 75 mM ammonium formate (pH 8.5). The hash (#) indicates an unknown non-proteinaceous compound. (**b**) Mass spectra obtained in the apices of the indicated peaks. After deconvolution, the degradation products (peak 1–4) were assigned based on their molecular mass and relative migration with respect to rhGH (peak 0). Reprinted with permission from ref. [16]

3.3.2 Analysis of Recombinant Human Interferon-β-1a by Sheath-Liquid CE-MS Using PB-DS-PB-Coated Capillaries

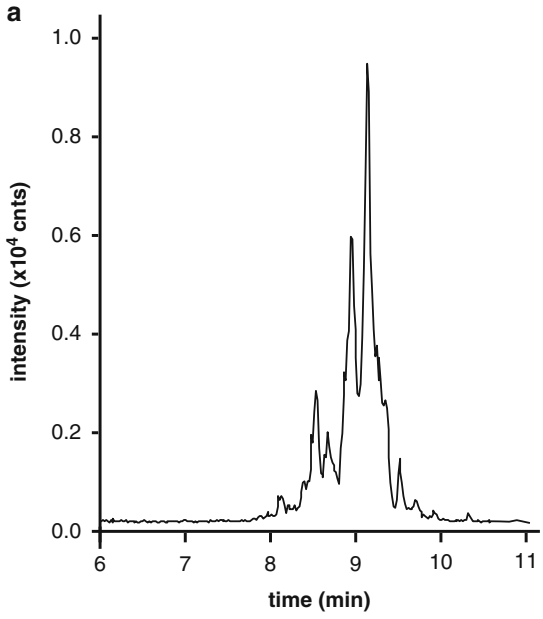
This example illustrates a sheath-liquid CE-MS methodology for the determination of a basic N-glycosylated protein, rhIFN-β. rhIFN-β is a 23-kDa protein, used for the treatment of multiple sclerosis. In order to avoid adsorption of the positively charged protein to the capillary wall, a positively charged coated capillary in combination with an acidic BGE is employed for separation.

1. Condition and coat the capillary as indicated in Subheadings 3.1, **step 1** and 3.2.2.
2. Perform the **steps 2–9** described in Subheading 3.3.1 using the following conditions: BGE composition: 50 mM acetic acid (pH 3.0); sheath liquid composition: acetonitrile–water–acetic acid (75/25/0.5, v/v/v)) at 2 μL/min; nebulizer pressure: 0.4 bar; dry gas: 4 L/min and 180 °C; separation voltage: −30 kV (*see* **Note 13**).
3. Monitor the base-peak electropherogram (BPE) obtained in the range of 1500–3000 *m/z*. Figure 3a shows the BPE obtained under these conditions.
4. Determine the mass of the observed peaks by deconvolution of the mass spectra. Perform spectral deconvolution as indicated in Subheading 3.4. Figure 3b lists the molecular masses and assigned glycan structure of rhIFN-β glycoforms obtained after deconvolution of the mass spectra (*see* **Note 14**).
5. At the end of the analysis, switch off the electrophoresis voltage and rinse the capillary with BGE (20 psi, 5 min).
6. Inject a new sample (**steps 2–4**) or clean the capillary with water (20 psi, 10 psi).

3.3.3 Analysis of Drug–Protein Conjugates by Sheathless CE-MS Using PEI-Coated Capillaries

Kinase-inhibiting drugs, such as erlotinib, can be bound to the renal carrier lysozyme, to enhance efficacy and improve the treatment of renal diseases using a BOCmethionine and a platinum-based linker (Fig. 4a). This example shows the use of a porous-tip sheathless CE-MS interface for the sensitive determination of erlotinib-LZM conjugates using an acidic BGE. To avoid adsorption of the positively charged proteins to the capillary wall, a PEI-coated capillary is employed.

1. Condition and coat the capillary as indicated in Subheadings 3.1, **step 2** and 3.2.3.
2. Install the nanospray end plate and gas diverter to allow nano-ESI and the XYZ-stage for the porous-tip capillaries.
4. Place the capillary in the XYZ-stage and position the spray tip at a distance of 3 mm with respect to the MS source (*see* **Note 15**).
5. Change the MS configuration to nanoESI.
6. Select the MS method and set the drying gas at 3 L/min N_2 and 180 °C and the scan range 300–3000 *m/z* (*see* **Note 16**).



b

migr. time (min)	mass	glycan
7.9	24424	2x 2x
8.0	24054	3x
8.1	23692	
8.3	23765	3x
8.3	23105	2x
8.5	23398	3x
8.5	23032	
8.8	22743	2x
9.1	22375	
9.5	22084	

Fig. 3 (**a**) BPE obtained during CE-TOF-MS of rhIFN-β (450 μg/mL) using a PB-DS-PB-coated capillary in combination with a BGE of 50 mM acetic acid (pH 3.0). (**b**) Migration time, molecular mass, and glycan composition for rhIFN-β glycoforms observed. Symbols: ●/●, hexose; ▲, fucose; ■, *N*-acetylhexosamine; ◆, sialic acid. Reprinted with permission from ref. [16]

7. Rinse the capillary and the conductive line with 100 mM acetic acid (pH 3.1) containing 5% (v/v) of isopropanol (100 psi, 10 min forward and 3 min reverse pressure).
8. To establish a stable spray and determine the optimum ESI voltage fill the capillary with BGE and apply a CE voltage of −30 kV across the capillary. Turn on the MS detection and gradually increase the ESI voltage until a background signal is observed. Then increase the ESI voltage by 200 V and wait until a stable baseline is observed. Set this value in the MS method.
9. Rinse the capillary and the conductive line with the BGE (100 mM acetic acid (pH 3.1) containing 5% (v/v) of isopropanol) (100 psi, 5 min forward and 3 min reverse pressure).
10. Inject the erlotinib-LZM conjugates by applying pressure (5 psi, 10 s) (*see* **Notes 17** and **18**).
11. Start the electrophoretic process by applying a voltage of −30 kV while using a capillary temperature of 20 °C.

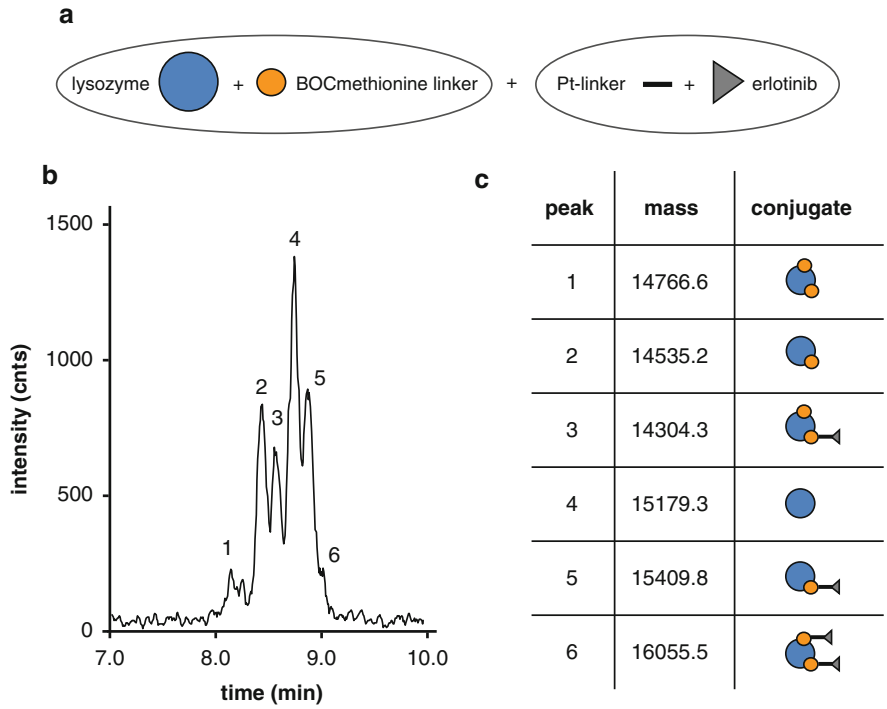


Fig. 4 (**a**) Schematic representation of the production of drug–protein conjugates. (**b**) BPE obtained with sheathless CE-MS for a erlotinib-ULS-BOCmet-LZM preparation using a PEI-coated porous-tip capillary and a 100 mM acetic acid (pH 3.1) BGE containing 5 % (v/v) of isopropanol. (**c**) Molecular mass and assigned conjugates obtained for peaks 1–6. Reprinted with permission from ref. [14]

12. Monitor the BPE obtained in the range of 1000–1800 *m/z*. Figure 4b shows the BPE obtained for the erlotinib-LZM conjugates under these conditions.
13. Determine the mass of the observed peaks by deconvolution of the mass spectra. Perform spectra deconvolution as indicated in Subheading 3.4. Molecular masses and assigned structure obtained for the erlotinib-LZM conjugates after deconvolution are shown in Fig. 4c.
14. At the end of the analysis, switch off the electrophoresis voltage and rinse the capillary and the conductive line with BGE (100 psi, 10 min forward and 3 min reverse pressure).
15. Inject a new sample (**steps 10–14**) or clean the capillary and the conductive line with water (100 psi, 10 min forward and 3 min reverse pressure).

3.3.4 Analysis of rhEPO by Sheathless CE-MS Using Neutrally Coated Capillaries

Recombinant human erythropoietin (rhEPO) is a glycoprotein hormone that is used for the treatment of anemia. The protein has three N-glycosylation sites (Asn^{24}, Asn^{38}, and Asn^{83}) and one O-glycosylation site (Ser^{126}). The total glycan content can make up about 40 % of the protein's molecular weight and introduces high

variation in both protein structure and mass. In this example, the use of a neutral porous-tip capillary in combination with an acidic BGE is described as a high resolution method for a more efficient separation for highly glycosylated proteins.

1. Condition the pre-coated neutral capillary as indicated in Subheading 3.1, **step 3**.
2. Perform the **steps 2–8** described in Subheading 3.3.3 using the following conditions: Drying gas, 1.2 L/min N_2 and 180 °C.
3. Rinse the capillary and the conductive line with the BGE (2 M acetic acid (pH 2.1)) (50 psi, 5 min forward and 3 min reverse pressure).
4. Inject rhEPO sample by applying pressure (5 psi, 10 s) and start the electrophoretic process by applying a voltage of 30 kV using a temperature of 20 °C.
5. Monitor the BPE obtained in the range 1000–3000 m/z. Figure 5a shows the BPE obtained for rhEPO under these conditions.
6. Determine the mass of the observed peaks by deconvolution of the mass spectra. Perform spectral deconvolution as indicated in Subheading 3.4. Figure 5b lists the molecular masses and assigned glycan structures of rhEPO glycoforms obtained after deconvolution of the mass spectra (*see* **Note 14**).
7. At the end of the analysis, switch off the electrophoresis voltage and rinse the capillary and the conductive line with BGE (50 psi, 5 min forward and 3 min reverse pressure).
8. Inject a new sample (**steps 4–8**) or clean the capillary and the conductive line with water (50 psi, 10 min forward and 3 min reverse pressure) and air (50 psi, 10 min forward and 3 min reverse pressure) and store the capillary at +4 °C.

3.4 Data Analysis Using Maximum Entropy Processing

ESI of proteins generates an envelope of multiply charged ions that are measured by their mass-to-charge ratio (m/z) in the mass spectrum. Transformation of the protein ESI spectrum into the mass spectrum of the neutral protein species (so-called deconvolution) is necessary to facilitate the interpretation of the MS results. Most MS software nowadays allows deconvolution of the mass spectrum by using dedicated algorithms. In the present examples, mass spectra were deconvoluted using the maximum entropy utility available in the data analysis software from Bruker Daltonics. For basic information about Maximum Entropy processing of proteins, the reader is referred to ref. [27]. For the present examples, deconvolution was performed as follows:

1. Select the peak of interest and average the mass spectra.
2. Choose the Maximum Entropy option in the deconvolution settings available in the software.

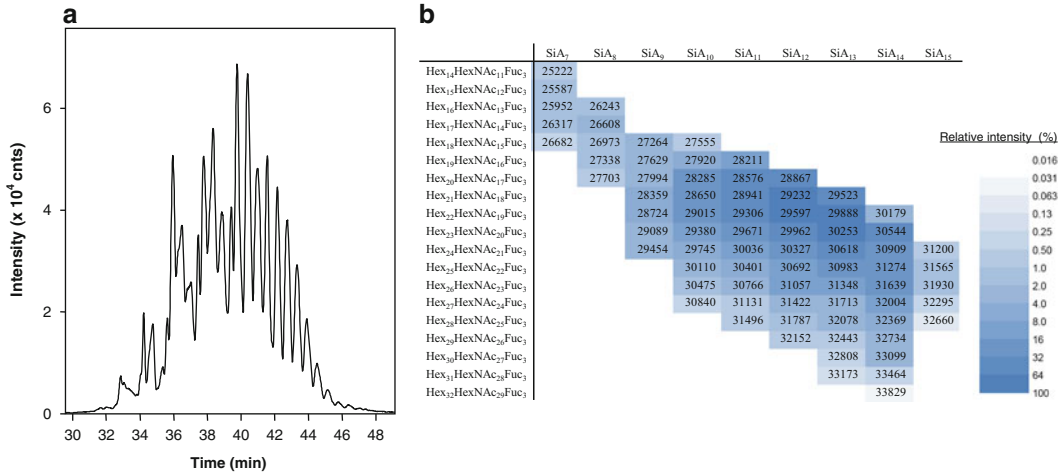


Fig. 5 (**a**) BPE obtained with sheathless CE-MS of rhEPO (200 μg/mL) using a neutral-coated porous-tip capillary and a 2 M acetic acid (pH 2.1) BGE. (**b**) Glycan composition and molecular mass of the rhEPO glycoforms observed. Reprinted with permission from ref. [24]

3. Set the mass range for the deconvolution and the following deconvolution parameters: data point spacing: 0.1 *m/z*; instrument resolving power: 10,000; normal resolution.
4. Process the average mass spectra to determine the intact masses of each protein in the selected peak.

3.5 Concluding Remarks

Overall, it can be concluded that CE-MS is a powerful tool for the analysis and characterization of intact proteins. CE-MS provides useful information on protein identity and purity, including modifications and degradation products. The presented examples show that CE-MS is especially suitable to probe protein modifications that lead to charge differences. For example, degradation products as a result of deamidation or sulfonic acid formation could be efficiently resolved from the parent compound. Also protein glycoforms which differ in the number of sialic acid groups could be separated. In case that modifications do not affect the overall protein charge, the high mass resolution and the accuracy of the TOF-MS still allowed assignment of components. Overall, CE-TOF-MS provides a highly promising means for the qualitative and quantitative assessment of the heterogeneity of (glycosylated) biopharmaceutical products.

4 Notes

1. Use the PEI solution for coating immediately.
2. Silanes are moisture-sensitive, keep the PEI bottle tightly close and store it in a cool (2–8 °C) and dry place.

3. Keep the capillary end outside the ionization source during the conditioning procedure in order to avoid the entrance of the nonvolatile components into the ionization source.
4. The first step of the capillary conditioning should be performed with the capillary tip immersed in methanol. For the rest of steps keep the capillary tip immersed in water.
5. Conductive line should be also conditioned. For this purpose, rinse the line for 3 min with MeOH followed by water by applying reverse pressure.
6. The porous tip should be immersed in water during this process.
7. If loss of peak efficiency and/or migration time reproducibility is observed most probably the coating is deteriorating. Rinse again with the last component of the coating (PVS or PB) (5 psi, 30 min) followed by deionized water (10 psi, 10 min) and BGE (20 psi, 10 min) and run the sample again. If efficiency is not recovered, a new coated capillary should be prepared.
8. The porous tip should be immersed in MeOH during the whole coating procedure.
9. The sheath liquid should be sufficiently conductive to ensure closure of the CE electric circuit.
10. Degassing the sheath liquid is recommended in order to eliminate the formation of air bubbles and to avoid drops in current.
11. For determination of intact proteins, a method optimized for high mass ranges should be used.
12. Note that depending on the inner diameter of the capillary, pressure applied should be modified to fill 1% of the capillary volume.
13. As the inner wall of the coated capillary is positively charged, polarity should be reversed to allow migration of the positive charged protein to the detector.
14. To assign the glycoforms mass differences between signals observed in the deconvoluted mass spectra were determined and compared with the calculated for each glycan based on the characteristic masses indicated in Table 1.
15. When the conductive line is filled with the BGE, drops can be formed at the end of the sprayer tip. To prevent aspiration of the conductive liquid to the MS inlet, do not position the sprayer tip closer than 2 mm from the MS source. This can be determined by applying ESI voltage while maintaining CE capillary flow at zero (no voltage or pressure). If ions are

Table 1
Symbol and average mass of fucose, *N*-acetylglucosamine, hexose, and sialic acid

		Average mass (Da)
	Fucose	146
	*N*-acetylglucosamine	203
	Mannose	162
	Galactose	162
	Sialic acid	291

observed in the total ion electropherogram (TIE), aspiration of the conductive liquid to the MS is taking place.

16. In nanoESI, nebulizer gas is not employed.
17. Note that due to the smaller inner diameter of the porous-tip capillaries (30 μm), the pressure applied for conditioning and injection should be higher than in conventional 50-μm capillaries (~2.7 times).
18. Isopropanol is not required for the ionization of the proteins in sheathless CE-MS but it provides slightly better signal intensities for the particular proteins analyzed.

References

1. Patrick JS, Lagu AL (2001) Review applications of capillary electrophoresis to the analysis of biotechnology-derived therapeutic proteins. Electrophoresis 22:4179–4196
2. Haselberg R, de Jong GJ, Somsen GW (2007) Capillary electrophoresis-mass spectrometry for the analysis of intact proteins. J Chromatogr A 1159:81–109
3. Haselberg R, de Jong GJ, Somsen GW (2011) Capillary electrophoresis-mass spectrometry for the analysis of intact proteins 2007-2010. Electrophoresis 32:66–82
4. Haselberg R, de Jong GJ, Somsen GW (2013) CE-MS for the analysis of intact proteins 2010-2012. Electrophoresis 34:99–112
5. Klampfl CW (2009) CE with MS detection: a rapidly developing hyphenated technique. Electrophoresis 30:S83–S91
6. Maxwell EJ, Chen DDY (2008) Twenty years of interface development for capillary electrophoresis-electrospray ionization-mass spectrometry. Anal Chim Acta 627:25–33
7. Brenner-Weiss G, Kirschofer F, Kuhl B et al (2003) Analysis of non-covalent protein complexes by capillary electrophoresis-time-of-flight mass spectrometry. J Chromatogr A 1009:147–153
8. Liang Z, Yang Q, Zhang WB et al (2003) Effects of experimental parameters on the signal intensity of capillary electrophoresis electrospray ionization mass spectrometry in protein analysis. Chromatographia 57:617–621
9. Huhn C, Ramautar R, Wuhrer M et al (2010) Relevance and use of capillary coatings in capillary electrophoresis-mass spectrometry. Anal Bioanal Chem 396:297–314
10. Moini M (2007) Simplifying CE-MS operation. 2. Interfacing low-flow separation techniques to mass spectrometry using a porous tip. Anal Chem 79:4241–4246
11. Zamfir AD (2007) Recent advances in sheathless interfacing of capillary electrophoresis and electrospray ionization mass spectrometry. J Chromatogr A 1159:2–13
12. Monton MRN, Terabe S (2005) Recent developments in capillary electrophoresis-mass spectrometry of proteins and peptides. Anal Sci 21:5–13

13. Haselberg R, Ratnayake CK, de Jong GJ et al (2010) Performance of a sheathless porous tip sprayer for capillary electrophoresis-electrospray ionization-mass spectrometry of intact proteins. J Chromatogr A 1217:7605–7611
14. Haselberg R, Harmsen S, Dolman MEM et al (2011) Characterization of drug-lysozyme conjugates by sheathless capillary electrophoresis-time-of-flight mass spectrometry. Anal Chim Acta 698:77–83
15. Stutz H (2009) Protein attachment onto silica surfaces—a survey of molecular fundamentals, resulting effects and novel preventive strategies in CE. Electrophoresis 30:2032–2061
16. Haselberg R, Brinks V, Hawe A et al (2011) Capillary electrophoresis-mass spectrometry using noncovalently coated capillaries for the analysis of biopharmaceuticals. Anal Bioanal Chem 400:295–303
17. Lucy CA, MacDonald AM, Gulcev MD (2008) Non-covalent capillary coatings for protein separations in capillary electrophoresis. J Chromatogr A 1184:81–105
18. Catai JR, Torano JS, de Jong GJ et al (2007) Capillary electrophoresis-mass spectrometry of proteins at medium pH using bilayer-coated capillaries. Analyst 132:75–81
19. Catai JR, Torano JS, Jongen PMJM et al (2007) Analysis of recombinant human growth hormone by capillary electrophoresis with bilayer-coated capillaries using UV and MS detection. J Chromatogr B 852:160–166
20. Haselberg R, de Jong GJ, Somsen GW (2009) Capillary electrophoresis of intact basic proteins using noncovalently triple-layer coated capillaries. J Sep Sci 32:2408–2415
21. Haselberg R, de Jong GJ, Somsen GW (2010) Capillary electrophoresis-mass spectrometry of intact basic proteins using polybrene-dextran sulfate-polybrene-coated capillaries: system optimization and performance. Anal Chim Acta 678:128–134
22. Haselberg R, van der Sneppen L, Ariese F et al (2009) Effectiveness of charged noncovalent polymer coatings against protein adsorption to silica surfaces studied by evanescent-wave cavity ring-down spectroscopy and capillary electrophoresis. Anal Chem 81:10172–10178
23. Balaguer E, Demelbauer U, Pelzing M et al (2006) Glycoform characterization of erythropoietin combining glycan and intact protein analysis by capillary electrophoresis electrospray time-of-flight mass spectrometry. Electrophoresis 27:2638–2650
24. Haselberg R, de Jong GJ, Somsen GW (2013) Low-flow sheathless capillary electrophoresis-mass spectrometry for sensitive glycoform profiling of intact pharmaceutical proteins. Anal Chem 85:2289–2296
25. Fretz MM, Dolman MEM, Lacombe M et al (2008) Intervention in growth factor activated signaling pathways by renally targeted kinase inhibitors. J Control Release 132:200–207
26. Prakash J, de Borst MH, Lacombe M et al (2008) Inhibition of renal rho kinase attenuates ischemia/reperfusion-induced injury. J Am Soc Nephrol 19:2086–2097
27. Green BN, Hutton T, Vinogradov SN (1996) Analysis of complex protein and glycoprotein mixtures by electrospray ionization mass spectrometry with maximum entropy processing. In: Chapman JR (ed) Methods of molecular biology, vol 61. Humana Press, London, pp 279–294

Chapter 4

Screening of Small Intact Proteins by Capillary Electrophoresis Electrospray Ionization-Mass Spectrometry (CE-ESI-MS)

Sabine Neuberger, Angelina Rafai, and Christian Neusüß

Abstract

Capillary electrophoresis (CE) has been shown to be a suitable separation technique for complex samples. Combined with electrospray ionization-mass spectrometry (ESI-MS), it is a powerful tool offering the opportunity of high selectivity and sensitivity combined with the possibility to identify and characterize intact proteins. In this protocol, we demonstrate a screening method for intact proteins based on capillary zone electrophoresis (CZE) separation coupled with online mass spectrometric detection. In order to avoid protein–wall interactions, a neutral coated capillary is used to create a universal method for proteins with both low and high electrophoretic mobilities. In addition, we show the successful validation and application of this screening method for a set of eight standard proteins and the glycoprotein erythropoietin.

Key words Capillary electrophoresis, Mass spectrometry, Electrospray ionization, Intact protein, Coating

1 Introduction

In the biopharmaceutical and clinical area, the characterization of proteins plays an important role. Especially for the analysis of intact proteins, capillary electrophoresis-mass spectrometry (CE-MS) becomes more and more important [1, 2]. Mass spectrometry (MS) enables the identification and characterization of the analyte. In combination with liquid chromatography (LC) or CE, it represents a highly suitable technique for the analysis of intact proteins. Compared to LC, CE provides a higher separation efficiency and more important a different selectivity (charge/size-ratio). Thus, it is often possible to separate protein isoforms like glycoforms. Moreover, proteins are easily charged in acidic background electrolyte (BGE) and therefore ideal for CE separation. For CE-MS coupling, electrospray ionization (ESI) is applicable due to the ionic character of the analyzed proteins.

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For CE with optical detection, a large number of highly selective and highly efficient protein separation systems have been developed based on capillary zone electrophoresis (CZE), capillary sieving electrophoresis (CSE), or capillary isoelectric focusing (cIEF). However, the BGEs contain nonvolatile constituents like ampholytes (cIEF), gels, and surfactants (CSE) or, e.g. ε-aminocaproic acid (CZE) interfering with ESI in a way that sensitive protein detection by MS is not possible. Using volatile electrolytes based on formate or acetate, it is possible to analyze proteins by CE-MS.

However, the analysis of intact proteins by CE is often interfered by the interaction of the protein and the negatively charged capillary wall [1–5]. These protein–wall interactions occur when bare-fused silica capillaries are used and affect the efficiency of the CE separation and the reproducibility of the migration time. In order to avoid the protein–wall interaction and to improve the performance of the CE separation, the application of highly acidic or highly alkaline BGE and/or the coating of the silica surface has been shown to be an effective option [1, 3–8]. Additives which are added to the BGE for a dynamic coating cause ion suppression in the ESI. Hence, for an efficient combination of CE and ESI-MS, only permanent or physically adsorbed coatings can be applied [1, 2, 5]. For the analysis of intact proteins by CE-MS, positively charged materials are most commonly applied [9–25]. Such positively charged materials are mostly combined with low-pH BGEs for the analysis of acidic and basic proteins. Cationic coating agents can be also used for the preparation of bi- or multilayer coated capillaries [10, 11, 26–28]. Compared to neutral coating materials [18, 25, 29–33] which suppress the electroosmotic flow (EOF) considerably, positively charged materials lead to a constant reversed EOF. The disadvantage of positive coating materials combined with low-pH BGEs is the high EOF which leads to a limitation of the CE resolution, since this is inversely proportional to the square root of the sum of the electroosmotic and analyte mobility [5]. Depending on the analyte mobility, it is important to optimize the EOF for each application in order to achieve an increase of resolution. It is not possible to find optimal EOF conditions applicable for a large set of varying analyte mobilities. Thus, for the screening of intact proteins neutral coated capillaries offer a broad applicability and a higher separation resolution for low mobility proteins [1, 5, 34].

This protocol presents a general CE-MS method for the characterization of a large variety of intact proteins [34]. The feasibility of the method is demonstrated on a mixture of eight standard proteins and erythropoietin (EPO). The standard proteins show a wide range of pI values (4.4–11.3) and hydrophobicities. Moreover, the different analytes cover the range of molecular weight up to about 33 kDa. The EPO molecule shows numerous naturally occurring glycoforms. These glycoforms show low mobilities at

acidic BGE. The separation and characterization of different glycoforms by CE-MS are challenging with respect to the minor mobility differences. Here, we demonstrate the applicability of different coating materials for the analysis of intact proteins. In addition, we discuss the influence of several parameters. Applying the given instructions it is possible to setup a system, which enables the screening of a large number of proteins by CE-ESI-MS.

2 Materials

2.1 Chemicals and Solutions

1. All solutions should be prepared using ultrapure water (18 M Ω cm at 25 °C).
2. Methanol, acetonitrile, 2-propanol, sodium hydroxide, and hydrochloric acid were used. All chemicals and solvents should be of highest purity, i.e. LC-MS-grade.
3. Lysozyme (from chicken egg white, purity not specified), β-lactoglobulin A (from bovine milk, ≥90%), myoglobin (from horse skeletal muscle, 95–100%), cytochrome c (from horse heart, ≥95%), trypsin inhibitor (from soybean, purity not specified), carbonic anhydrase (from human erythrocytes, purity not specified), and RNase A and B (from bovine pancreas, ≥80%) were used as standard proteins.
4. UltraTrol™ low normal (LN), a dynamic coating based on derivatized polyacrylamide, was purchased from Target Discovery (Palo Alto, CA, USA). The permanently Guarant™ coated capillaries are commercial available (Alcor BioSeparations, Palo Alto, CA, USA).
5. Recombinant EPO beta (NeoRecormon®, Hoffmann-La Roche, Basel, Switzerland) was provided as an injection solution containing 10,000 international units of EPO.
6. ES Tuning Mix was obtained from Agilent Technologies (Waldbronn, Germany). The solution was diluted 1:50 in 95% acetonitrile (ACN) and 5% water before use. The diluted solution could be used for several weeks when stored at 2–8 °C.
7. BGE for CE separation: 0.5 M acetic acid (HAc) for standard proteins and 1.0 M HAc for EPO.
8. Sheath liquid (SL): 1:1 (v/v) 2-propanol:ultrapure water, 1% HAc (*see* **Note 1**).
9. For ultrafiltration filter devices with a 10 kDa cutoff were used.
10. Before usage the different solutions should be mixed well and degassed in an ultrasonic bath for at least 10 min (*see* **Note 2**).
11. In order to prevent cross contaminations, it is recommended to use always the same bottle for the same solutions. Moreover, the storage of all solutions is best in high-quality bottles (e.g. borosilicate glass).

2.2 Instrumentation

2.2.1 CE

1. In principle all CE instruments can be used for the separation. However, it should be possible to guide the capillary out of the instrument and to apply a stable power up to +30 kV at the inlet vial at both polarities. Here, a HP $_{3D}$CE was used (Agilent Technologies, Waldbronn, Germany).
2. Fused-silica capillaries of 50 μm ID × 360 μm OD were supplied by Polymicro Technologies (Phoenix, AZ, USA). The capillary length used typically is about 60 cm (*see* **Note 3**).
3. In order to ensure a flat surface, the cutting of the capillaries should be controlled under a microscope (*see* **Note 4**).
4. About 1 cm of the polyimide layer is removed at the capillary outlet by flame and cleaned with 1:1 (v/v) 2-propanol:ultrapure water (*see* **Note 5**).
5. The installation of the capillary in the CE instrument is performed according to the respective manual of the used instrument.
6. The instrument is controlled by ChemStation software (Agilent Technologies, Waldbronn, Germany).

2.2.2 Coupling

1. A coaxial sheath liquid interface (Agilent Technologies, Waldbronn, Germany) is used for the CE-MS coupling. Sheath liquid is delivered by a 5 mL gas-tight syringe using a Cole-Parmer syringe pump (Vernon Hill, IL, USA).
2. The MS and the CE are controlled by the respective software. A trigger cable between the two instruments provides the connection by transferring start/stop signals. Using this coupling between both instruments, unattended sequences are possible by proper timing of the CE and MS methods, respectively.

2.2.3 Mass Spectrometry

1. Different types of mass spectrometers may be used for the protein analysis. Here, we used a micrOTOF-Q time-of-flight mass spectrometer from Bruker Daltonik (Bremen, Germany) (*see* **Notes 6** and **7**).
2. The micrOTOF-Q is equipped with an analog-to-digital converter (ADC) for a discrimination-free measurement of the isotope pattern. This allows the usage of singly charged ions for the calibration of highly charged molecules like intact proteins.

3 Methods

For the characterization of proteins, different CZE-MS methods can be used. Formic acid-based systems are suitable for the separation of many peptides and proteins [35, 36]. Especially for the separation of the different glycoforms of complex glycoproteins, the application of HAc as BGE yields better results.

3.1 Sample Treatment

The analysis typically requires a total protein concentration of about 1 μg/μL for the characterization of large and/or complex proteins. Less complex or smaller proteins can be analyzed at the low to medium ng/μL level.

The possible injection and separation are influenced by the sample matrix. For proteins which are highly soluble in water, a pre-cleaning step by ultrafiltration is recommended (*see* **Note 8**).

1. Stock solutions (3 μg/μL) were prepared by dissolving the different proteins in water. The analyzed protein sample was a mixture of the single stock solutions, containing 63 ng/μL of lysozyme and β-lactoglobulin A, 125 ng/μL of ribonuclease (RNase) A and B, 188 ng/μL of trypsin inhibitor and carbonic anhydrase each.
2. In general, protein solutions should be prepared freshly. Depending on the stability of the protein, the prepared solutions can be stored at 2–8 °C for several days. For certain proteins a longer storage at –20 °C is possible.
3. The EPO syringe content (0.6 mL) was purified by ultrafiltration using a 10,000 Da cutoff membrane. Thereafter, EPO was lyophilized by a vacuum centrifuge and redissolved in 20 μL of water leading to a final concentration of ≈4 μg/μL (10,000 IU/83 μg EPO).

3.2 Capillary Zone Electrophoresis

The CE separation is the most critical step after the instrument setup. Hence, careful attention has to be paid to the following steps:

1. All solutions and BGEs are degassed before usage by ultrasonication.
2. For the conditioning and coating procedure the outlet of the capillary is left outside the sprayer (*see* **Note 9**).
3. Before the capillary is installed into the sprayer, new capillaries are conditioned by flushing (≥1 bar) with methanol (5 min), 1 M NaOH (20 min) followed by flushing with water for 10 min.
4. Coating of the capillary is performed by flushing the capillary with undiluted UltraTrol™ LN (5 min), water (2 min), and BGE (5 min) (*see* **Notes 10**–**12**). Prior using Guarant capillaries were only flushed by water. The trimethoxysilylpropyl(polyethylenimine) coating (PEI, Gelest, Morrisville, PA, USA) was prepared according to the US Patent 6923895 B2 applying one layer.
5. The capillary is now coupled to the MS according to the instructions for the sheath liquid sprayer (*see* Subheading 3.3).
6. The performance and the stability of the system are tested by applying a voltage of +30 kV for 10 min. Over the whole period the current should be stable within ±5 μA. For a 60 cm long capillary, a current between 11 and 15 μA is expected when 1 M HAc is used as BGE (*see* **Note 13**).

7. Prior to the CE separation the pre-conditioning step is performed by exchanging the volume of the capillary at least twice with BGE (flushing for, e.g. 2 min with approximately 1 bar).
8. The sample is injected hydrodynamically by application of 50 mbar for 24 s (*see* **Note 14**).
9. After sample injection the analysis is started. Therefore, a separation voltage of +30 kV is applied. A slightly lower and decreasing current is acceptable at the beginning. This effect is caused by the low conductivity of the sample zone (*see* **Note 15**).
10. Post-conditioning is performed by flushing the capillary for several minutes with BGE by approximately 1 bar.

3.3 CE-MS Coupling

1. In order to prevent MS contamination, it is important that the pre-conditioning, coating, and flushing with BGE are finished before the capillary is placed into the sprayer. Moreover, the capillary should be removed from the sprayer for each re-conditioning step.
2. The installation of the capillary into the sprayer is performed according to the manual provided by Agilent Technologies. It is important to take the right capillary position into account to ensure a stable spray during the analysis. For the positioning a magnifier should be used. The capillary is positioned well when only a hardly visible section of the capillary looks out of the sprayer needle [37].
3. Prior the syringe filling with sheath liquid it is necessary to degas the solution by ultrasonication. The syringe is coupled via standard PEEK tubing and fittings to the sprayer. A small air bubble at the end of the syringe damps potential pulsation which might alter the ESI process and could damage the syringe pump over time. The sheath liquid is supplied by a syringe pump with a flow rate of 3 μL/min.

3.4 Mass Spectrometry

1. For positive electrospray ionization, a negative potential at the inlet of the MS is applied (−4.5 kV).
2. A nebulizer gas pressure of 0.2 mbar is applied to assist the spraying process (*see* **Note 16**).
3. Dry gas is delivered at a flow rate of 4 L/min and a temperature of 170 °C. Higher temperatures can cause instabilities of the CE-MS interface.
4. The ion optics are optimized to the highest possible intensity in the mass range of m/z 700–3000 by direct infusion of a 50-fold dilution of ES Tuning Mix with a flow rate of 4 μL/min.
5. The same solution and flow rate are used for the mass calibration of the MS. The calibration was performed at least once a day.
6. Spectra are acquired by summarizing 5000/10,000 single spectra, leading to a time resolution of 1 s/2 s.

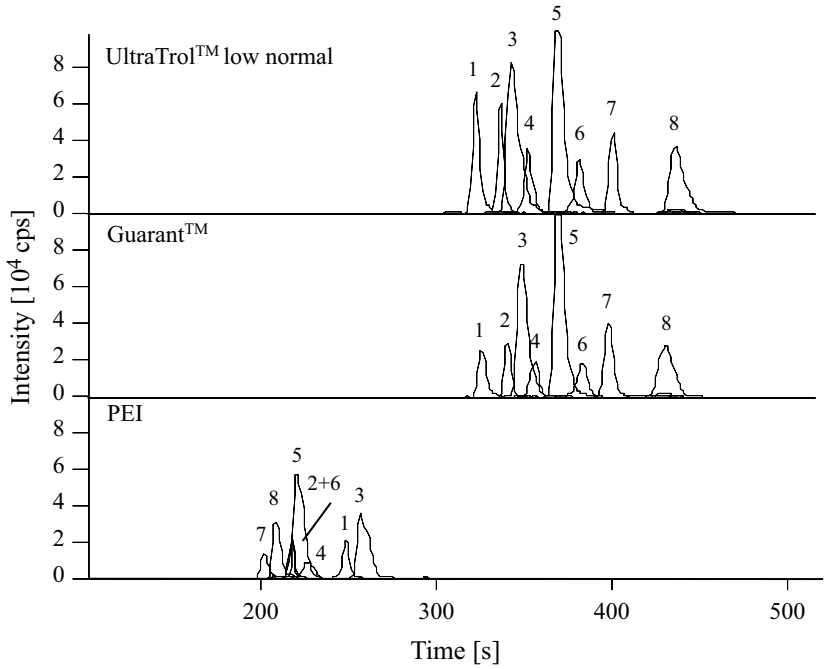

Fig. 1 Comparison of the separation of model proteins using 0.5 M HAc as BGE and three different coated capillaries. For the eight standard proteins ion traces were created. The three most abundant charge states of each protein were summed up to an extracted ion electropherogram (EIE). The ion traces correspond as follows to the measured model proteins: (1) lysozyme, (2) β-lactoglobulin A, (3) cytochrome c, (4) RNase A, (5) myoglobin, (6) RNase B, (7) trypsin inhibitor, and (8) carbonic anhydrase

7. The "maximum entropy" algorithm in DataAnalysis™ version 4.0 (Bruker Daltonics) is used for charge deconvolution of the averaged protein mass spectra.
8. The masses and charge distributions of the proteins, which are used for trace extraction, are calculated using the "Isotope Pattern" tool of Bruker Daltonics.
9. Three major ions from the charge distribution of each protein are summed up to one extracted ion electropherogram (EIE) (*see* Fig. 1) (*see* **Note 17**).

Figure 2a shows a mass spectrum derived from the analysis shown in Fig. 1. The spectrum shows an example for the charge distribution obtained from RNase A. The deconvoluted spectrum is shown in Fig. 2b (*see* **Note 18**).

3.5 Validation

1. For validation, the intra- and interday precisions of the migration times and the peak areas are determined.
2. In order to calculate the limits of detection (LODs) and limits of quantitation (LOQs), the ion traces created by the m/z extraction of the three abundant charge states are used. LODs are based on S/N = 3.

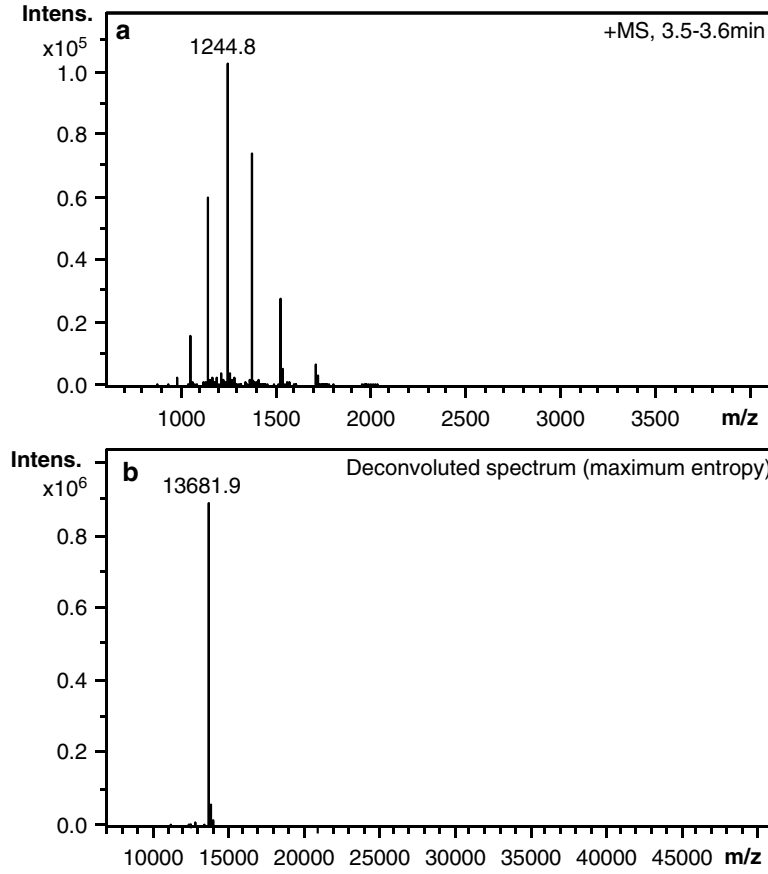

Fig. 2 Mass spectrum (**a**) and corresponding charge deconvoluted spectrum (**b**) of RNase A from the analysis shown in Fig. 1

For the standard proteins, the obtainable intra- and interday precisions regarding the migration times are in the range of 0.3–2.4%. Regarding the peak areas, relative standard deviations (RSDs) of 9.0–23.8% are reached. The LODs are in the range of 0.06–0.81 ng/μL. LOQs (S/N = 10) in the range of 0.20–2.5 ng/μL were achieved. In principle, there should be a linear correlation for protein concentrations in the range of 0.20–375 ng/μL covering at least two orders of magnitude. The linearity can be tested by the Mandel test [38].

4 Notes

1. In order to avoid weak ionization efficiency, it may help to add an ionization modifier. Add 0.1–1% (v/v) acetic acid or formic acid to the sheath liquid in case of positive ionization in protein analysis.

2. Degassing of all solutions is required in order to avoid gas bubble formation inside the capillary. For the removal of gases, it is necessary to degas all electrolytes and solvents at least 5–10 min. Gas bubbles introduce practical problems like unstable currents and current breakdowns. Thus, if problems with unstable currents occur, it is necessary to check if all solutions are degased well.
3. Depending on the used instruments, a longer capillary length is necessary due to the instruments dimension. The increase of the length leads to a squared increase of the separation time, while theoretically the separation is not influenced by the length. In practice, the use of a shorter capillary length shows only minor influence on the separation as long as a minimum length of about 50–60 cm is used. For commercial CE-MS applications, this minimum length is the shortest possible.
4. Proper (flat) cutting is important to achieve orthogonal and flat tips especially for the outlet of the capillary. A "nose" of glass prolonging over the cut should be avoided. Either a rotating diamond tool or a ceramic cutting plate can be used. By the use of a ceramic cutter, it is important to apply as low pressure as possible to the capillary. After cutting the capillary is broken by pulling at both ends while slightly beginning to bend.
5. In order to avoid swelling of the polyimide layer in the presence of organic solvents in the sheath liquid it is recommended to remove it.
6. In principle, MS instruments where ESI-HV is applied at the MS inlet and not at the ESI spray needle are preferred for CE-MS coupling. The spray needle in instruments, provided by Bruker Daltonics and Agilent Technologies, is grounded. The decoupling of the two HV circuits of CE and ESI is beneficial for separation and stable electrospray conditions.
7. TOF or Q-TOF mass spectrometers offer high resolution and high mass accuracy which are important especially in protein analysis. These instruments perfectly measure the narrow peaks typically obtained in CE separations due to the high acquisition speed. MS/MS experiments (CID in QqTOF) may help for the identification. However, electron transfer dissociation (ETD) provides often higher information for intact proteins.
8. A pre-cleaning step is recommended in the case where higher amounts of salts, detergents, or other small molecules are present. Ultrafiltration can be used for preconcentration as well.
9. Contamination of the interface or the MS with nonvolatile substances which were used for conditioning or coating procedures should be strictly avoided. Thus, it is best that the capillary is not installed in the sprayer during these procedures.

10. For reproducible CE analysis, the EOF control is essential. In general, CE-MS coupling requires an EOF towards the capillary outlet to achieve a stable CE-MS interface. In order to enhance the analyte resolution in the CE-MS coupling, it is necessary to optimize the EOF. The resolution in CE-ESI-MS can be described by the following equation [5, 34]:

$$R = \frac{1}{4\sqrt{2}} \times \Delta\mu \sqrt{\frac{U}{D \times \left(\bar{\mu} + \mu_{eo}\right)}}$$

where $\Delta\mu$ is the effective mobility difference of two analytes, U the applied voltage, D the diffusion coefficient, $\bar{\propto}$ the mean electrophoretic mobility of the analytes of interest, and μ_{eo} the mobility of the EOF. Hence, the resolution and the analysis time will be maximal when the sum of μ_{eo} and $\bar{\propto}$ is minimal (absolute vector value).

In case of neutral coated capillaries the EOF is nearly zero. Thus, the term $\left(\bar{\mu} + \mu_{eo}\right)$ is dominated by the analyte mobility and a higher resolution is obtained for slow migrating analytes. If a charged coated capillary producing a significant EOF is used, two cases are possible. On the one hand, for co-electroosmotic migration, meaning that the EOF and the analyte mobilities are in the same direction, a high value for the sum of μ_{eo} and $\bar{\propto}$ is obtained and the achieved resolution is low. On the other hand, if the EOF and the analyte mobility are in the opposite direction (counter-electroosmotic migration) low values for the term $\bar{\propto} + \propto_{eo}$ are obtained and the achievable resolution is higher. A very high resolution can be obtained when the electrophoretic migration and the electroosmotic transport are nearly balanced, requiring a dedicated coating. However, this setup is not applicable for a universal (screening) method.

An example for the separation of a mixture of model proteins on different coated capillaries is shown in Fig. 1. A cationic coating based on polyethylenimine (PEI) and two neutral coated capillaries (UltraTrol™ LN and Guarant™) are used. The electropherograms show that the neutral coated capillaries lead to higher resolutions. Moreover, the achieved resolutions for both neutral coatings are comparable.

The comparison of the mean electrophoretic mobilities for the different model proteins and an example of EPO with the EOF mobilities of three different coatings is summarized in Table 1. The values clearly show that a low normal or low reversed EOF is necessary to achieve a good resolution during the separation of low mobility proteins (e.g. EPO). Conversely, in order to obtain a good separation of high mobility proteins (e.g. lysozyme), a low normal or high reversed EOF is necessary. A coating which causes a low

Table 1
Mean electrophoretic mobilities of model proteins and EPO isoforms compared to the EOF mobilities of different coatings

	μ (10^{-9} $m^2/V/s$)
EOF, UltraTrol™ LN	1.3
EOF, Guarant™	0.3
EOF, PEI	–92
Lysozyme	35.2
β-Lactoglobulin A	32.7
Cytochrome c	32.0
RNase A	30.6
Myoglobin	29.2
RNase B	27.8
Trypsin inhibitor	26.7
Carbonic anhydrase	24.1
EPO isoforms	9.6–13.0

The used BGE is composed of 0.5 M HAc, (pH 2.5) for model proteins and 1 M acetic acid, pH 2.4 for EPO experiments

reversed EOF is not applicable as it is not able to transport the fast migrating protein toward the MS detector. In general, a neutral coating is universal and therefore more suitable as a general method for the analysis of intact proteins.

11. In order to avoid any contamination with other coatings used on the same instrument, all vials should be used only for a certain application. In addition, the level of the vial containing the coating solution should be low enough so that the electrodes are not contaminated. Moreover, it is necessary to clean the electrodes before using the CE with other coatings or with non-coated capillaries. Alternatively, the coating procedure can be performed outside the CE by using a syringe pump.
12. In order to improve the coating stability and the separation efficiency, the capillary should be recoated every 5–10 runs. For this purpose, the capillary has to be removed from the sprayer and flushed for 5 min with 3 M HCl followed by flushing 5 min with water prior to the mentioned coating procedure.
13. The actual current value depends on the capillary length and may differ slightly. The current is also higher for aged or re-used BGEs. In this case, the electrolyte should be prepared freshly.

14. The injected sample plug should reach a maximum of 1–2% of the total capillary volume. Higher injection volumes may be performed (up to 10% of the capillary) if a sample of low ionic strength is used.
15. During the separation, it is recommendable to monitor the current. Different reasons for current instabilities are possible. Sheath liquid entering the capillary can cause current breakdown or a continuous decrease of the current. Height differences of the CE and the MS should be eliminated to avoid siphoning. Alternatively, it is possible to slightly increase the nebulizer pressure or a low pressure can be applied during separation. These two latter parameters are useful in order to find out if siphoning is the reason for the instable current. Generally, errors in the sheath liquid setup (air bubbles, low flow, etc.) may lead to low current in the CE. In case a current breakdown is observed during the run (after injection) the incompatibility of the sample and the BGE due to differences in the pH (acidification of a basic system causes gas bubbles, i.e. CO_2) or an organic solvent content (different solubility of air) is often the reason.
16. In general, the applied nebulizer pressure should be low in order to avoid a siphoning effect in the capillary. The height of the applied pressure depends on the positioning of the metal needle inside the sprayer and could be increased slightly (!) in order to achieve a stable spray.
17. The influence of the number of charge states being considered for the ion trace extraction (three vs. all) on the peak area precisions was investigated. For the proteins, similar values were obtained when three or all charge states were used [34].
18. In the case of glycoproteins like EPO the deconvoluted spectrum is used to determine the differences in glycosylation by mass differences. In general, for these isotopically non-resolved proteins the mass precision is better than 1 Da when calibration is performed internally or directly before/after the analysis (within ± 30 min).

References

1. Haselberg R, de Jong GJ, Somsen GW (2013) CE-MS for the analysis of intact proteins 2010–2012. Electrophoresis 34:99–112
2. Pioch M, Bunz S-C, Neusüß C (2012) Capillary electrophoresis/mass spectrometry relevant to pharmaceutical and biotechnological applications. Electrophoresis 33:1517–1530
3. Lucy CA, MacDonald AM, Gulcev MD (2008) Non-covalent capillary coatings for protein separations in capillary electrophoresis. J Chromatogr A 1184:81–105
4. Stutz H (2009) Protein attachment onto silica surfaces—a survey of molecular fundamentals, resulting effects and novel preventive strategies in CE. Electrophoresis 30:2032–2061
5. Huhn C, Ramautar R, Wuhrer M, Somsen GW (2010) Relevance and use of capillary coatings in capillary electrophoresis–mass spectrometry. Anal Bioanal Chem 396:297–314
6. Dolník V (2008) Capillary electrophoresis of proteins 2005–2007. Electrophoresis 29: 143–156

7. El Rassi Z (2010) Electrophoretic and electrochromatographic separation of proteins in capillaries: an update covering 2007–2009. Electrophoresis 31:174–191
8. Haselberg R, de Jong GJ, Somsen GW (2011) Capillary electrophoresis-mass spectrometry for the analysis of intact proteins 2007–2010. Electrophoresis 32:66–82
9. Moini M (2010) Metal displacement and stoichiometry of protein-metal complexes under native conditions using capillary electrophoresis/mass spectrometry. Rapid Commun Mass Spectrom 24:2730–2734
10. Haselberg R, Brinks V, Hawe A, de Jong GJ, Somsen GW (2011) Capillary electrophoresis-mass spectrometry using noncovalently coated capillaries for the analysis of biopharmaceuticals. Anal Bioanal Chem 400:295–303
11. Haselberg R, de Jong GJ, Somsen GW (2010) Capillary electrophoresis-mass spectrometry of intact basic proteins using Polybrene–dextran sulfate-polybrene-coated capillaries: system optimization and performance. Anal Chim Acta 678:128–134
12. Haselberg R, Ratnayake CK, de Jong GJ, Somsen GW (2010) Performance of a sheathless porous tip sprayer for capillary electrophoresis-electrospray ionization-mass spectrometry of intact proteins. J Chromatogr A 1217:7605–7611
13. Haselberg R, Harmsen S, Dolman MEM, de Jong GJ, Kok RJ, Somsen GW (2011) Characterization of drug-lysozyme conjugates by sheathless capillary electrophoresis—time-of-flight mass spectrometry. Anal Chim Acta 698:77–83
14. van der Meel R, Oliveira S, Altintas I, Haselberg R, van der Veeken J, Roovers RC, van Bergen en Henegouwen PMP, Storm G, Hennink WE, Schiffelers RM, Kok RJ (2012) Tumor-targeted nanobullets: anti-EGFR nanobody-liposomes loaded with anti-IGF-1R kinase inhibitor for cancer treatment. J Control Release 159:281–289
15. Ongay S, Martín-Álvarez PJ, Neusüß C, de Frutos M (2010) Statistical evaluation of CZE-UV and CZE-ESI-MS data of intact α-1-acid glycoprotein isoforms for their use as potential biomarkers in bladder cancer. Electrophoresis 31:3314–3325
16. Ongay S, Neusüβ C (2010) Isoform differentiation of intact AGP from human serum by capillary electrophoresis–mass spectrometry. Anal Bioanal Chem 398:845–855
17. Neusüß C, Demelbauer U, Pelzing M (2005) Glycoform characterization of intact erythropoietin by capillary electrophoresis-electrospray-time of flight-mass spectrometry. Electrophoresis 26:1442–1450
18. Balaguer E, Neusüss C (2006) Glycoprotein characterization combining intact protein and glycan analysis by capillary electrophoresis-electrospray ionization-mass spectrometry. Anal Chem 78:5384–5393
19. Sanz-Nebot V, Benavente F, Giménez E, Barbosa J (2005) Capillary electrophoresis and matrix-assisted laser desorption/ionization-time of flight-mass spectrometry for analysis of the novel erythropoiesis-stimulating protein (NESP). Electrophoresis 26: 1451–1456
20. Simó C, Elvira C, González N, San Román J, Barbas C, Cifuentes A (2004) Capillary electrophoresis-mass spectrometry of basic proteins using a new physically adsorbed polymer coating. Some applications in food analysis. Electrophoresis 25:2056–2064
21. Ullsten S, Zuberovic A, Wetterhall M, Hardenborg E, Markides KE, Bergquist J (2004) A polyamine coating for enhanced capillary electrophoresis-electrospray ionization-mass spectrometry of proteins and peptides. Electrophoresis 25:2090–2099
22. Elhamili A, Wetterhall M, Arvidsson B, Sebastiano R, Righetti PG, Bergquist J (2008) Rapid capillary electrophoresis time-of-flight mass spectrometry separations of peptides and proteins using a monoquaternarized piperazine compound (M7C4I) for capillary coatings. Electrophoresis 29: 1619–1625
23. Yu B, Cong H, Liu H, Li Y, Liu F (2005) Ionene-dynamically coated capillary for analysis of urinary and recombinant human erythropoietin by capillary electrophoresis and online electrospray ionization mass spectrometry. J Sep Sci 28:2390–2400
24. Balaguer E, Neusüß C (2006) Intact glycoform characterization of erythropoietin-α and erythropoietin-β by CZE-ESI-TOF-MS. Chroma 64:351–357
25. Balaguer E, Demelbauer U, Pelzing M, Sanz-Nebot V, Barbosa J, Neusüß C (2006) Glycoform characterization of erythropoietin combining glycan and intact protein analysis by capillary electrophoresis—electrospray—time-of-flight mass spectrometry. Electrophoresis 27:2638–2650
26. Catai JR, Torano JS, de Jong GJ, Somsen GW (2007) Capillary electrophoresis-mass spectrometry of proteins at medium pH using bilayer-coated capillaries. Analyst 132:75–81
27. Catai JR, Sastre Toraño J, Jongen PMJM, de Jong GJ, Somsen GW (2007) Analysis of recombinant human growth hormone by capillary electrophoresis with bilayer-coated capillaries using UV and MS detection. J Chromatogr B 852:160–166

28. Sanz-Nebot V, Balaguer E, Benavente F, Neusüß C, Barbosa J (2007) Characterization of transferrin glycoforms in human serum by CE-UV and CE-ESI-MS. Electrophoresis 28:1949–1957
29. Giménez E, Benavente F, Barbosa J, Sanz-Nebot V (2008) Analysis of intact erythropoietin and novel erythropoiesis-stimulating protein by capillary electrophoresis-electrospray-ion trap mass spectrometry. Electrophoresis 29:2161–2170
30. Aguilar C, Hofte AJP, Tjaden UR, van der Greef J (2001) Analysis of histones by on-line capillary zone electrophoresis–electrospray ionisation mass spectrometry. J Chromatogr A 926:57–67
31. Thakur D, Rejtar T, Karger BL, Washburn NJ, Bosques CJ, Gunay NS, Shriver Z, Venkataraman G (2009) Profiling the glycoforms of the intact α subunit of recombinant human chorionic gonadotropin by high-resolution capillary electrophoresis–mass spectrometry. Anal Chem 81:8900–8907
32. Hoffmann T, Martin MM (2010) CE-ESI-MS/MS as a rapid screening tool for the comparison of protein–ligand interactions. Electrophoresis 31:1248–1255
33. Taichrib A, Pelzing M, Pellegrino C, Rossi M, Neusüß C (2011) High resolution TOF MS coupled to CE for the analysis of isotopically resolved intact proteins. J Proteomics 74:958–966
34. Taichrib A, Pioch M, Neusüß C (2012) Toward a screening method for the analysis of small intact proteins by CE-ESI-TOF MS. Electrophoresis 33:1356–1366
35. Bateman K, Kelly J, Thibault P, Ramaley L, White R (2003) Glycoprotein analysis by capillary zone electrophoresis-electrospray mass spectrometry. In: Thibault P, Honda S (eds) Capillary electrophoresis of carbohydrates. Humana Press, Totowa, NJ, pp 219–239
36. Hernández-Borges J, Neusüß C, Cifuentes A, Pelzing M (2004) On-line capillary electrophoresis-mass spectrometry for the analysis of biomolecules. Electrophoresis 25:2257–2281
37. Ohnesorge J, Neusüß C, Wätzig H (2005) Quantitation in capillary electrophoresis-mass spectrometry. Electrophoresis 26:3973–3987
38. Brüggemann L, Quapp W, Wennrich R (2006) Test for non-linearity concerning linear calibrated chemical measurements. Accred Qual Assur 11:625–631

Chapter 5

Online Capillary IsoElectric Focusing-ElectroSpray Ionization Mass Spectrometry (CIEF-ESI MS) in Glycerol–Water Media for the Separation and Characterization of Hydrophilic and Hydrophobic Proteins

Meriem Mokaddem, Fanny d'Orlyé, and Anne Varenne

Abstract

Capillary isoelectric focusing (CIEF) is a high-resolution technique for the separation of ampholytes, such as proteins, according to their isoelectric point. CIEF coupled online with MS is regarded as a promising alternative to 2-D PAGE for fast proteome analysis with high-resolving capabilities and enhanced structural information without the drawbacks of conventional slab-gel electrophoresis. However, online coupling has been rarely described, as it presents some difficulties. A new methodology for the online coupling of CIEF with electrospray ionization mass spectrometry (ESI-MS) has been developed in glycerol–water media. This new integrated methodology provides a mean for the characterization of a large number of hydrophilic and hydrophobic proteins.

Key words Amphoteric compound, Protein, Isoelectric focusing, Mass spectrometry, Ampholytes, Glycerol anticonvective medium, Isoelectric point and mass determination

1 Introduction

CIEF, which is the second most popular CE mode, allows separating amphoteric compounds, like proteins, according to their isoelectric point (p*I*). CIEF is considered to provide the highest resolving power of all CE modes, even separating protein isoforms with single amino acid substitution, deletion, or insertion, and affords a resolution of around 0.02 pH unit. The focusing effect concentrates proteins by a factor of 50–100 [1], making even diluted samples accessible to MS detection. CIEF coupled online with MS is regarded as a promising alternative to 2-D PAGE for fast proteome analysis, providing information on isoelectric point and molecular mass (*M*r), with high resolving capabilities and enhanced structural information, omitting drawbacks of

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conventional slab-gel electrophoresis, such as restrictions in sensitivity as well as tedious staining procedures [2]. For now only few CIEF-MS coupling methodologies have been described, mainly offline involving MALDI-MS detection [3–6]. Indeed online CIEF-MS coupling presents some difficulties, among which (1) maintaining the electrical continuity required for the electrophoretic separation, (2) the compatibility of the composition of the fluid arising from the separation capillary with MS detector, i.e. the anti-convective gel and the ampholytes needed in CIEF, and (3) the chemical and thermal resistance of the inner wall of the separation capillary within the interface, the capillary being currently a neutral coated capillary so as to avoid protein adsorption and strongly reduce EOF.

A new methodology for the online coupling of capillary isoelectric focusing (CIEF) with electrospray ionization mass spectrometry (ESI-MS) has been developed in glycerol–water media [7]. This improved protocol provides (1) the electric continuity during the whole analysis by a discontinuous filling of the capillary in two continuous zones containing catholyte and protein–ampholyte mixture respectively, (2) the use of an anticonvective medium, i.e. glycerol/water mixture, compatible with MS detection and as an aid to hydrophobic protein solubilization, and (3) the use of unmodified bare fused-silica capillaries, as the glycerol/water medium strongly reduces EOF and avoids protein adsorption. Focusing was performed in positive polarity and further cathodic mobilization was achieved by both voltage and pressure application in order to maintain pH gradient during mobilization till the detector. This new integrated protocol should be an easy and effective additional tool in the field of proteome analysis, providing a mean for the characterization of a large number of hydrophilic and hydrophobic proteins, for their isoelectric point and molecular mass determinations.

2 Materials

Prepare all solutions using ultrapure water (produced by purifying deionized water to attain a sensitivity of 18 M Ω cm at 25 °C) and analytical grade reagents. Prepare all reagents at room temperature and store them at 4 °C or −20 °C, according to cases. Diligently follow all waste disposal regulations when disposing waste materials.

2.1 Protein–Ampholyte Mixture

1. Separation medium: glycerol/water (30:70, v/v). Degas the mixture by ultrasound for 3 min and store at 4 °C (*see* **Note 1**).
2. Carrier ampholytes mixture (Pharmalyte, Beckman Coulter): store at 4 °C (*see* **Note 2**).

3. Model proteins: ribonuclease A (Rnase, pI=9.45, Mr 13 676 Da), carbonic anhydrase II (CA II, pI=5.9, Mr 29 080 Da), β-lactoglobulin A (β-Lac, pI=5.1, Mr 18 357 Da), α-chymotrypsinogen A type II (α-Tryp, bovine pancreas pI=8.3, Mr 25 661 Da), myoglobin (Myo, horse heart pI=6.3, Mr 16 946 Da), trypsin inhibitor (TI, type I-S soy bean pI=4.5, Mr 19 982 Da). Store the lyophilized model proteins at −20 °C (*see* **Note 3**).
4. Stock solutions of protein: dissolve in the separation medium at a concentration in the mg/mL range. Aliquote (2 µL) and store at −20 °C (*see* **Note 4**).
5. Protein–ampholyte mixture: add carrier ampholyte mixture (1.5% v/v) in the separation medium glycerol/water (*see* **Note 5**). Add proteins at concentrations allowing their detection (classically from 0.01 to 0.5 mg/mL: 0.1 to 15 µM) (*see* **Note 6**). Prior to capillary loading, vortex for 10 s and centrifuge for 10 min at 3000 × *g* to homogenize and avoid air bubbles.

2.2 Anolyte and Catholyte Composition (See Note 7)

1. Anolyte solution (*see* **Note 8**): 50 mM formic acid/1 mM glutamic acid (pH 2.35) in the separation medium. Add 0.096 mL of a 98% formic acid solution (1.22 density) in 50 mL separation medium. Weigh 7.35 mg glutamic acid. Dissolve it in the latter solution. Degas by ultrasound for 5 min and store at 4 °C (*see* **Note 9**).
2. Catholyte solution (*see* **Note 10**): 100 mM ammonia/1 mM lysine (pH 10.6) in the separation medium. Add 0.373 mL of a 25% ammonia solution (0.91 density) in 50 mL of separation medium. Weigh 9.13 mg lysine. Dissolve it in the latter solution. Degas by ultrasound for 5 min and store at 4 °C (*see* **Note 11**).

2.3 Capillary Preparation and Storage

Bare-fused silica capillary: 100 cm total length, 50 µm internal diameter, 360 µm outer diameter. Condition new capillaries by successive flushes with 1 M and 0.1 M NaOH and then with water under a pressure of 93.5 kPa for 15 min each. Between runs, rinse with water for 3 min, 10 mM HCl for 10 min and finally water for 5 min. Dry by air when not in use (*see* **Note 12**).

2.4 Sheath Liquid Composition

Sheath liquid: MeOH–H_2O mixture (50:50 v/v) containing 1% v/v acetic acid. Mix 50 mL ultrapure water and 50 mL methanol of Normapure grade. Add 1 mL acetic acid in the latter solution and degas by ultrasound for 5 min before storing at 4 °C.

3 Methods

Carry out all procedures at room temperature unless otherwise specified.

3.1 CIEF Protocols (See Note 13)

1. Analytical protocols including capillary conditioning, sample introduction and voltage application, were automatized and thus realized in one step (*see* **Note 14**). Set the temperature in the capillary cartridge at 25 °C. To realize a partial filling of the capillary, for 100 cm length flush the capillary for 3 min at 94.5 kPa with the catholyte solution and 56 s at 94.5 kPa with the protein–ampholyte mixture so that the capillary length containing this latter solution is equal to 40 cm (taking into account the viscosity of the separation medium). Place the anolyte in the inlet vial. Apply a voltage of 30 kV for 6 min to achieve the step of protein focusing. Mobilize the pH gradient zone to cathodic side under a 5 kPa pressure, while maintaining focusing voltage applied, until all proteins are detected (*see* Fig. 1) (*see* **Note 15**).

3.2 Electrospray Interface (ESI)

Electrospray interface consists in a triple coaxial tube nebulizer held at ground potential. Deliver the coaxial sheath liquid at a flow rate of 6 µL/min with an isocratic pump equipped with a splitter (1:100). Set the nebulizing gas consisting in nitrogen at 100 °C (pressure 55 kPa) (*see* **Note 16**).

3.3 Electrospray Interface Parameters

Connect the orthogonal ESI with a single quadrupole mass spectrometer in the positive ionization mode. Use nitrogen as drying gas at 350 °C (flow rate, 7 L/min). Set ESI and skimmer voltage at 3500 and 10 V, respectively. Set peak width and dwell time at 0.3 min and 880 ms, respectively (*see* **Note 17**).

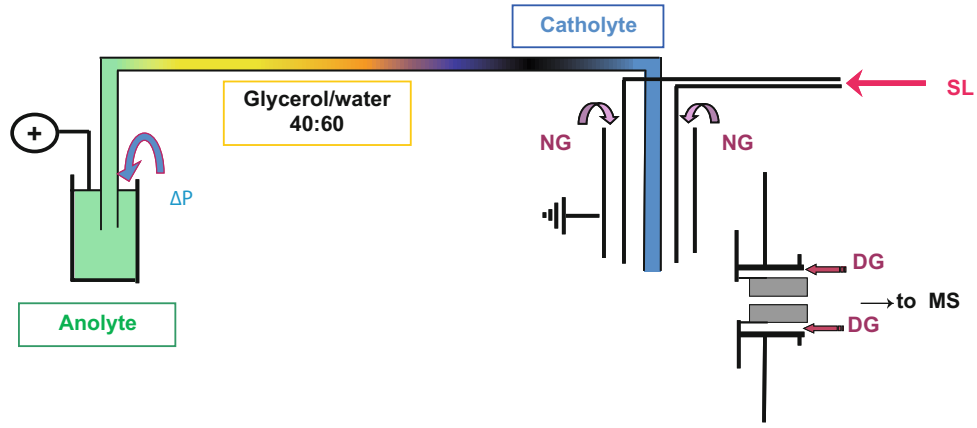

Fig. 1 Schematic representation of the online glycerol/water CIEF-ESI-MS protocol. *SL* sheath liquid, *NG* nebulizing gas, *DG* drying gas

3.4 Data Treatment and Calibration Curve (See Fig. 2)

Record both the overall scan mode MS signal (m/z 1000–2500) (*see* Fig. 2a) and the extracted ion current (EIC) signal (*see* Fig. 2c) (*see* **Note 18**) for each protein. Perform signal acquisition of anolyte acid and catholyte base in the selected ion monitoring mode (SIM) at m/z 148 (*see* Fig. 2b). Extract the mass spectra of the proteins (*see* **Note 19** and Fig. 2).

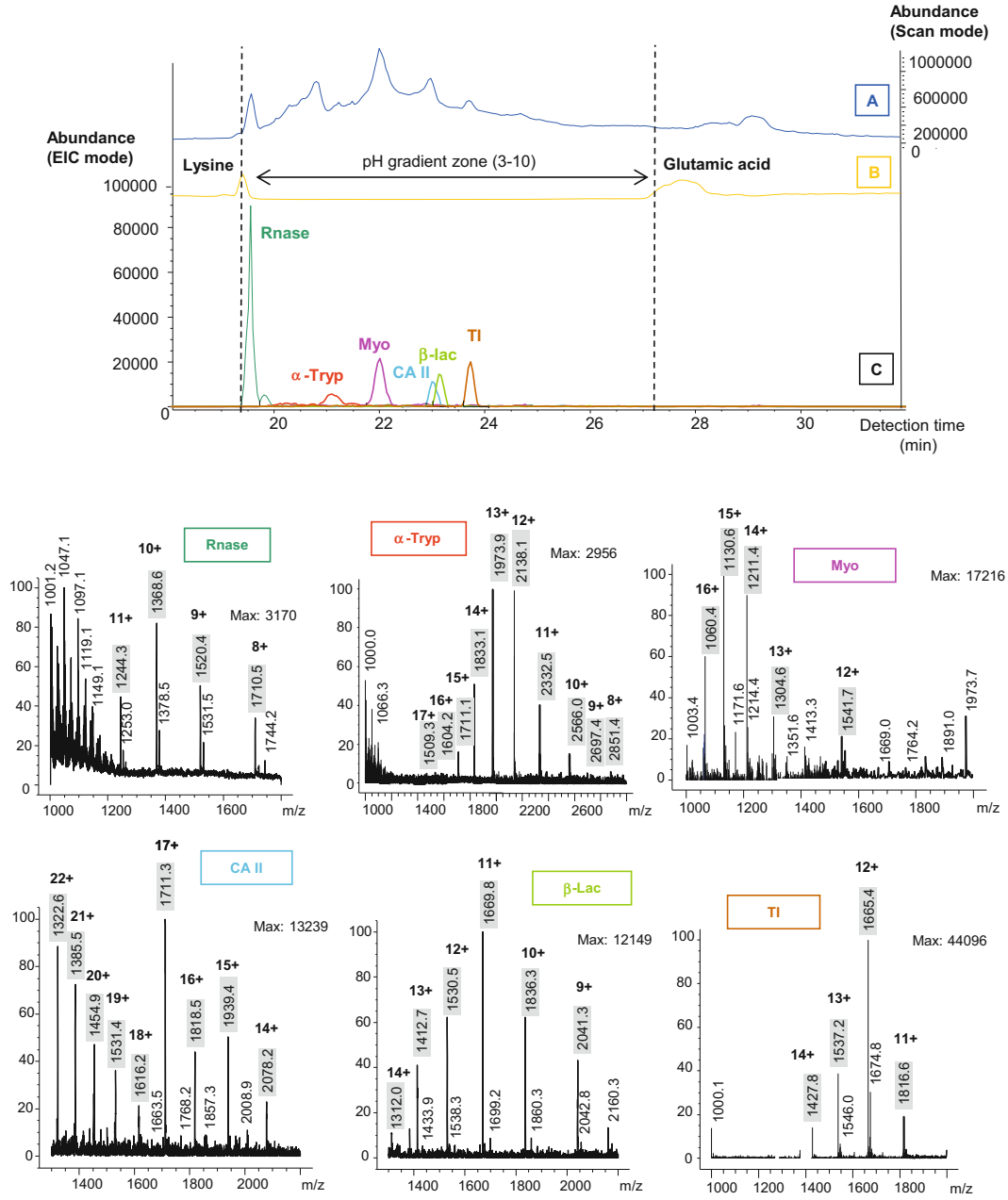

Fig. 2 Online CIEF-ESI-MS and extracted MS spectra of six model proteins. (**a**) Scan mode (m/z 1000–2500) electropherogram; (**b**) SIM mode (m/z 148) electropherogram; (**c**) EIC mode electropherogram: Rnase (m/z 1369), α-Tryp (m/z 2139), Myo (m/z 1211), CA II (m/z 1940), β-Lac (m/z 2041), TI (m/z 1666). Mass spectra of the six model proteins extracted from the average scan under the peak in (**a**). Adapted from ref. [11], with permission

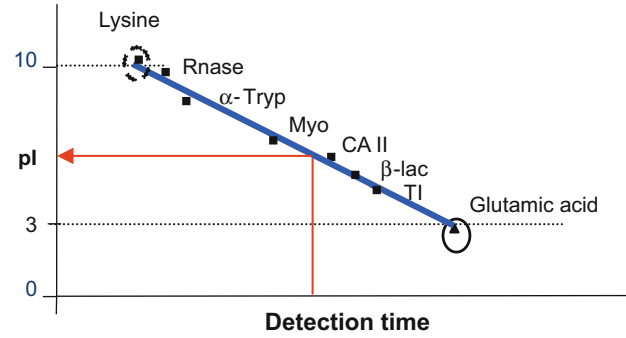

Fig. 3 Protein p*I* as a function of detection time

Perform the calibration curve by plots of *pIs* of model proteins in terms of their detection time (*see* Fig. 3). Determine the p*I* and *Mr* of the protein of interest using calibration curve and mass spectrum, respectively.

Characterize an unknown mixture probably containing a highly basic cytokine human interferon-gamma (IFN-γ) [8] and its variants. Identify IFN-γ with a 0.05 p*I* and 18 907.5 ± 1.2 *Mr*. Identify eleven variants with p*I* in the range from 9.6 to 9.99 and *Mr* in the range from 16,095 ± 1.2 to 16,886.2 ± 1.3.

4 Notes

1. Thanks to its high viscosity, glycerol, which is known to better solubilize and stabilize hydrophobic proteins, can both play the role of anticonvective medium (to establish and stabilize the pH gradient) and allow employing bare silica capillaries, as it considerably reduces EOF and protein adsorption on capillary walls. Glycerol is furthermore MS compatible.
2. The carrier ampholytes mixture is a commercial mixture. When applying an electric field, they allow to create a pH gradient zone in the capillary (*see* Fig. 3). This pH gradient can be classically in the 3–10 pH range, or implemented with ampholyte mixtures of narrower pH gradient (4–6 or 9–10 for example, according to protein p*I*) for a better p*I* determination (*see* Fig. 4a, b).
3. Model proteins allow to calibrate the pH gradient within the separation so as to allow the accurate p*I* determination for an unknown protein. It is observed that myoglobin alters after a 1-day dilution in the glycerol/water medium, leading to an unexplained shift in *Mr* from 16,946 to 17,564 Da.
4. An aliquot of proteins is taken from the freezer, each day, and thawed at room temperature to prepare the protein–ampholyte mixture.

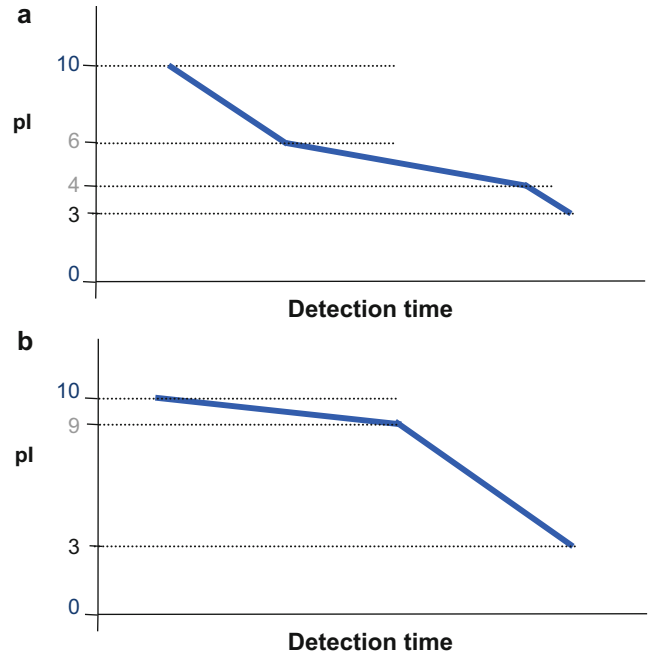

Fig. 4 Protein p*I* as a function of detection time. pH gradient (pH 3–10) implemented with ampholyte mixture of narrower pH gradient. (**a**) 4–6 and (**b**) 9–10

5. Ampholyte concentration in the protein–ampholyte mixture strongly influences peak intensity, protein charge state on the mass spectrum and detection time. The existence of an intensity maximum indicates that a compromise should be found between separation performances, favored by high ampholyte concentration, and detection performances, favored by low ampholyte concentration.
6. Although the m/z values of ampholytes are mostly beyond the m/z range of most proteins [9], their presence in the sprayed solution causes a reduction in the MS signal intensity due to ion suppression [10–13] and background signal, and decreases the mass resolution.
7. Anolyte and catholyte allow to complete the pH gradient zone at its extremities. They therefore allow the good pH gradient stability during the focusing and mobilization steps. They are prepared in the glycerol/water mixture (30:70 v/v). By doing so, anolyte and catholyte viscosity are the same as that of the separation electrolyte, which prevents dispersion during the mobilization step due to viscosity differences.
8. Anolyte has to be compatible with MS detection. To visualize the limits of the pH gradient zone at the acidic side during separation, glutamic acid ($M_r = 146.2$, p$Ka = 2.19$), which is easily detectable by MS, is added in small quantity in the anolyte.

9. Different acidic solutions were tested [14–17], the separation being discussed with respect to the time span of the pH gradient zone and resolution. 50 mM formic acid/1 mM glutamic acid (pH 2.35) is retained as anolyte as it provides short analysis time, for similar resolutions, and low MS noise.
10. Catholyte has to be compatible with MS detection. To visualize the limits of the pH gradient zone at the basic side during separation, lysine ($M_r = 147.1$, $pKa = 10.53$), which is easily detectable by MS, is added in small quantity in the catholyte.
11. Different basic solutions were tested [14–17], the separation being discussed with respect to the time span of the pH gradient zone and resolution. 100 mM ammonia/1 mM lysine (pH 10.6) is retained as catholyte as it provides short analysis time, for similar resolutions, and low MS noise.
12. So as to suppress or at least lower EOF, CIEF is usually performed in fused-silica capillaries coated with hydrophilic polymers, such as polyacrylamide or polyvinylalcohol [10, 11, 18–20]. However, few wall modifications are compatible with MS interfacing conditions so far as heating at the last few centimeters of the capillary outlet by the nebulizing gas may lead to coating degradation and risk of bleeding [17, 21–24].
13. This method allows to obtain in a single capillary the successive zones of catholyte, protein–ampholyte mixture, and anolyte so as to generate the pH gradient. The outlet vial is replaced by the ESI interface, so as to perform a real unattended "online" coupling providing better efficiency and reproducibility. Therefore, the electric field is maintained between the focusing and the mobilizing steps (to preserve separation efficiency).
14. The analysis time depends on the respective zone lengths in the capillary, the focusing time and the applied voltage and pressure during mobilization step.
15. This method avoids the moving ion boundary phenomenon occurring when catholyte is introduced in the SL. Ions from the SL enter the capillary, shift the pH gradient, and make the proteins migrate toward the ESI source [1]. This moving ionic boundary, however, could also contribute to a loss in resolution and an inversion of migration order, but the problems could be remediated by applying a gravity-induced hydrodynamic flow during mobilization [2, 25].
16. The protrusion of the CE capillary from the interface needle has serious consequences on the spray performances [26–30], i.e. spray stability and ionization efficiency or yield, as it influences the mixing quality between SL and BGE. The signal intensity goes past a sharp maximum for a protruding length of 0.25 ± 0.05 mm from the SL tube (with nebulizing gas pressure set at 55 kPa and SL flow rate at 6 mL/min) [31]. The contact between the pH gradient zone and the catholyte is not located at the interface, which improves the electrospray stability.

17. The general configuration selected during this study for CIEF-ESI-MS is as follows: (1) SL helps for positive ionization of the proteins, as the positive ionization mode is more sensitive than the negative ionization one; (2) the discontinuous electrolyte filling and the inlet vial containing the anolyte allow an actual online coupling. Furthermore, the fact that the length of the protein–ampholyte mixture zone is shorter than the total capillary length permits shorter detection times for the pH gradient zone, and thus globally shorter analysis time.
18. SIM/Scan modes: For the model proteins, the EIC (extracted ion current) signals are two to three times lower than the ones obtained in SIM mode; nevertheless, the EIC mode allows to identify an unlimited number of known proteins in a single run, provided that there is no ionization suppression for proteins detected simultaneously. This online protocol thus appears as a powerful tool for fast identification of a large number of proteins, through their characterization in terms of p*I* and molecular mass.
19. Addition of glycerol to electrospray of protein solutions results in an increase in the number of charge states in the gas phase, related to the high surface tension of glycerol [32]. Furthermore, such addition at levels up to 45 % does not measurably affect the intensity of ESI signal. When glycerol–water mixtures with 10–40 % glycerol are employed, no clogging of the MS transfer capillary is observed and no specific washing has to be performed between runs.

References

1. Yang L, Lee CS, Hofstadler SA et al (1998) Capillary isoelectric focusing-electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry for protein characterization. Anal Chem 70:3235–3241
2. Jensen PK, Pasa-Tolic L, Peden KK et al (2000) Mass spectrometric detection for capillary isoelectric focusing separations of complex protein mixtures. Electrophoresis 21: 1372–1380
3. Minarik M, Foret F, Karger BL (2000) Fraction collection in micropreparative capillary zone electrophoresis and capillary isoelectric focusing. Electrophoresis 21:247–254
4. Foret F, Muller O, Thoren J et al (1996) Analysis of protein fractions by micropreparative capillary isoelectric focusing and matrix-assisted laser desorption time-of-flight mass spectrometry. J Chromatogr A 716:157–166
5. Chartogne A, Gaspari M, Jespersen S et al (2002) On-target fraction collection for the off-line coupling of capillary isoelectric focusing with matrix-assisted laser desorption/ionization mass spectrometry. Rapid Commun Mass Spectrom 16:201–207
6. Yu W, Yan L, Deng C et al (2006) Comprehensive two-dimensional separation in coupling of reversed-phase chromatography with capillary isoelectric focusing followed by MALDI-MS identification using on-target digestion for intact protein analysis. Electrophoresis 27:2100–2110
7. Mokadddem M, Gareil P, Varenne A (2009) Online CIEF-ESI-MS in glycerol-water media with a view to hydrophobic protein applications. Electrophoresis 30:4040–4048
8. Przybylski C, Mokaddem M, Prull-Janssen M et al (2015) On-line capillary isoelectric focusing hyphenated to native electrospray ionization mass spectrometry for the characterization of interferon-γ and variants. Analyst 140:543–550
9. Righetti PG, Simo C, Sebastiano R et al (2007) Carrier ampholytes for IEF, on their fortieth anniversary (1967–2007), brought to trial in court: the verdict. Electrophoresis 28:3799

10. Tang Q, Harrata AK, Lee CS (1995) Capillary isoelectric focusing-electrospray mass spectrometry for protein analysis. Anal Chem 67:3515–3519
11. Zhou F, Johnston MV (2004) Protein characterization by on-line capillary isoelectric focusing, reversed-phase liquid chromatography, and mass spectrometry. Anal Chem 76:2734–2740
12. Storms HF, Van der Heijden R, Tjaden UR et al (2004) Capillary isoelectric focusing-mass spectrometry for shotgun approach in proteomics. Electrophoresis 25:3461–3467
13. Storms HF, Van der Heijden R, Tjaden UR et al (2005) Optimization of protein identification from digests as analyzed by capillary isoelectric focusing-mass spectrometry. J Chromatogr B 824:189–200
14. Clarke NJ, Naylor S (2002) Capillary isoelectric focusing-mass spectrometry: analysis of proteins mixtures human body fluids. Biomed Chromatogr 16:287–297
15. Lamoree MH, Tjaden UR, Van der Greef J (1997) Use of microdialysis for the on-line coupling of capillary isoelectric focusing with electrospray mass spectrometry. J Chromatogr A 777:31–39
16. Yang L, Lee CS, Hofstadler SA et al (1998) Characterization of microdialysis acidification for capillary isoelectric focusing-microelectrospray ionization mass spectrometry. Anal Chem 70:4945–4950
17. Ullsten S, Zuberovic A, Wetterhall M et al (2004) A polyamine coating for enhanced capillary electrophoresis-electrospray ionization-mass spectrometry of proteins and peptides. Electrophoresis 25:2090–2099
18. Wei J, Yang L, Harrata AK, Lee CS (1998) High resolution analysis of protein phosphorylation using capillary isoelectric focusing-electrospray ionization-mass spectrometry. Electrophoresis 19:2356–2360
19. Martinovic S, Pasa-Tolic KL, Masselon C et al (2000) Characterization of human alcohol dehydrogenase isoenzymes by capillary isoelectric focusing-mass spectrometry. Electrophoresis 21:2368–2375
20. Jensen PK, Harrata AK, Lee CS (1998) Monitoring protein refolding induced by disulfide formation using capillary isoelectric focusing-electrospray ionization mass spectrometry. Anal Chem 70:2044–2049
21. Li M, Liu L, Wu JT et al (1997) Use of a polybrene capillary coating in capillary electrophoresis for rapid analysis of hemoglobin variants with on-line detection via an ion trap storage/reflectron time-of-flight mass spectrometer. Anal Chem 69:2451–2456
22. Kelly JF, Ramaley L, Thibault P (1997) Use of a polybrene capillary coating in capillary electrophoresis for rapid analysis of hemoglobin variants with on-line detection via an ion trap storage/reflectron time-of-flight mass spectrometer. Anal Chem 69:51–60
23. Simo C, Elvira C, Gonzalez N et al (2004) Capillary electrophoresis-mass spectrometry of basic proteins using a new physically adsorbed polymer coating. Some applications in food analysis. Electrophoresis 25: 2056–2064
24. Soga T, Ueno Y, Naraoka H et al (2002) Simultaneous determination of anionic intermediates for bacillus subtilis metabolic pathways by capillary electrophoresis electrospray ionization mass spectrometry. Anal Chem 74:2233–2239
25. Zhang C-X, Xiang F, Pasa-Tolic L et al (2000) Stepwise mobilization of focused proteins in capillary isoelectric focusing mass spectrometry. Anal Chem 72:1462–1468
26. Ohnesorge J, Sänger-van de Griend C, Wätzig H (2005) Quantification in capillary electrophoresis-mass spectrometry: long- and short-term variance components and their compensation using internal standards. Electrophoresis 26:2360–2375
27. Geiser L, Rudaz S, Veuthey JL (2003) Validation of capillary electrophoresis-mass spectrometry methods for the analysis of a pharmaceutical formulation. Electrophoresis 24:3049–3056
28. Nunez O, Moyano E, Galceran MT (2002) Capillary electrophoresis-mass spectrometry for the analysis of quaternary ammonium herbicides. J Chromatogr A 974:243–255
29. Sanz-Nebot V, Benavente F, Balaguer E et al (2003) Capillary electrophoresis coupled to time of flight-mass spectrometry of therapeutic peptide hormones. Electrophoresis 24:883–891
30. Nilsson SL, Bylund D, Jörnten-Karlsson M et al (2004) A chemometric study of active parameters and their interaction effects in a nebulized sheath-liquid electrospray interface for capillary electrophoresis-mass spectrometry. Electrophoresis 25:2100–2107
31. Mokaddem M, Gareil P, Belgaied JE et al (2008) A new insight into suction and dilution effects in capillary electrophoresis coupled to mass spectrometry via an electrospray ionization interface. Part I-suction effect. Electrophoresis 29:1957–1964
32. Iavarone AT, Williams ER (2002) Supercharging in electrospray ionization: effects on signal and charge. Int J Mass Spectrom 219:63–72

Chapter 6

On-Line Solid-Phase Extraction Capillary Electrophoresis Mass Spectrometry for Preconcentration and Clean-Up of Peptides and Proteins

Fernando Benavente, Silvia Medina-Casanellas, Estela Giménez, and Victoria Sanz-Nebot

Abstract

One of the major drawbacks of capillary electrophoresis (CE) and other microscale separation techniques, for the analysis of low abundant peptides and proteins in complex samples, are the poor concentration limits of detection. Several strategies have been developed to improve CE sensitivity. Here, we describe an on-line solid-phase extraction capillary electrophoresis mass spectrometry method with a commercial C18 sorbent for clean-up and preconcentration of neuropeptides from highly diluted biological samples.

Key words Analyte concentrator, Capillary electrophoresis, Clean-up, In-line, Mass spectrometry, Microcartridge, On-line, Peptides, Preconcentration, Sensitivity, Solid phase extraction

1 Introduction

1.1 Solutions for the Sensitivity Issue in Capillary Electrophoresis

The benefits of capillary electrophoresis (CE) for the analysis of peptides and proteins are renowned [1–4]. However, like in many other microscale separation techniques, concentration sensitivity is poor because of the very small sample injection volumes (typically 1–2 % of the capillary volume) [1–12]. Today, the most effective approaches to decrease limit of detection (LOD) in CE include the use of selective and sensitive detectors (e.g. LIF and MS detection), or on-column preconcentration with electrophoretic [5–7] and chromatographic techniques [8–12]. Sensitivity enhancement with electrophoretic preconcentration techniques (e.g. different modes of stacking, dynamic pH junction, transient-isotachophoresis (^{t}ITP), sweeping, etc.) can be considerable, but it is extremely analyte- and sample matrix-dependent. Furthermore, in most cases the maximum injected sample is limited to one capillary volume and the gain in loadability is achieved at expense of losing separation resolution, as a large portion of the separation capillary is used

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to load the sample [5–7]. The topic has been comprehensively reviewed in capillary and microchip formats [5–7], and the reported sensitivity enhancement for peptides and proteins is typically between 10- and 100-fold, with some authors rarely reporting values up to 500- to 1000-fold (or higher) for certain peptides [13–16]. ^{t}ITP [17–22], dynamic pH junction [23–25] and selective electrokinetic injection [26, 27] are the preferred approaches with on-line MS detection, because of the compatibility with volatile solutions [21, 28, 29]. Furthermore, ^{t}ITP is especially useful for analyzing high ionic strength complex samples (e.g. biological samples) [5–7]. Results with these preconcentration approaches can be further improved in some cases when combining several electrophoretic techniques (e.g. electrokinetic supercharging (EKS) [14] or dynamic pH junction-sweeping [15]), or even better, because of higher versatility, off-line or on-line chromatographic techniques [18, 19].

1.2 Coupling Solid-Phase Extraction to Capillary Electrophoresis

In general, chromatographic preconcentration techniques, such as solid-phase extraction (SPE), allow the analysis of larger sample volumes [8–12, 18, 19]. There are different strategies to combine SPE with CE [8–12]. Off-line or at-line SPE-CE require extensive and time-consuming manual handling or an automated device transferring the SPE extracts to the CE system, respectively, because there is no direct connexion between both techniques. On-line coupling can be achieved via a valve or a tee connector, but it is in general preferred, for simplicity and performance, to directly insert the microcartridge or analyte concentrator in the separation capillary. This last configuration is referred as in-line for some authors because the microcartridge is integrated within the CE separation capillary [11, 12].

1.3 On-Line Solid-Phase Extraction Capillary Electrophoresis

In the most typical configuration for on-line SPE-CE, the extraction microcartridge is placed near the inlet of the separation capillary and contains a sorbent (C18, C8, C2, ion exchange, immobilized metal affinity chromatography (IMAC), antibody, lectin, aptamer, etc.) that retains the target analyte from a large volume of sample [8–12, 18, 19, 30–36]. After rinsing to eliminate non-retained molecules and to fill the capillary with background electrolyte (BGE), the retained analyte is eluted in a smaller volume of appropriate solution, resulting in sample clean-up and concentration enhancement, before the electrophoretic separation and detection. A number of different microcartridge designs have been described [8–12]. We have widely applied particle-packed microcartridges with frits to prevent sorbent particle bleeding [8, 18, 30–34]. However, some authors prefer fritless microcartridges with membranes, filters, monoliths, magnetic particles, molecularly imprinted polymers or particles with an appropriate size, to prevent backpressure, EOF disturbance and bubble formation issues that may occur if frits are improperly installed [35, 36]. With

regard to the sorbents, SPE-CE has been extensively explored using the reversed-phase silica-based sorbent particles typically used in off-line SPE (e.g. C18) [8, 18, 30–32, 36]. These sorbents are commercially available, have been extensively used in the analysis of peptides, proteins and small organic molecules and can be purchased at a reasonable price. However, what is even more important is that they provide a large active surface area, without interfering with the on-line electrophoretic separation or detection. Thus, for example, with on-line MS detection, ionic strength and volatility of the solutions can be easily optimized to obtain the best preconcentration factors while preventing ion suppression and salt build-up in the detector [8, 18, 30–32, 36]. This is not the case with other sorbents (e.g. immunoaffinity, IMAC, etc.), that require very specific conditions for the extraction which are scarcely compatible with on-line MS detection [33, 34]. The major drawback of reversed-phase sorbents is their limited selectivity, which hinders the analysis of complex samples such as biological fluids if a selective detection, such as MS, is not applied for the reliable identification of the detected compounds. In a previous work, we demonstrated that C18-SPE-CE–MS is useful to lower the LODs for the analysis of opioid peptides in plasma samples up to 1000-fold compared to the values previously obtained by CE–MS with a sheath-flow interface [30, 31]. These LODs can be further decreased combining an electrophoretic preconcentration step by ^{t}ITP (^{t}ITP-C18-SPE-CE–MS) [18], or using a sheathless CE-MS nanoelectrospray interface [36].

In this chapter, we describe the method that we routinely follow to construct, and use in a commercial CE instrument, capillaries for SPE-CE-MS using a C18 sorbent for clean-up and preconcentration of neuropeptides (methionine encephalin, Met, endomorphin1, End and dynorphin A (1–7), DynA) from standard solutions and plasma samples. The method is meant to be a guide to expand the applicability of this unique approach, giving the clues to overcome the main limitations found by non-initiated users, which are often related to the lack of clear guidelines and specific training.

2 Materials

Prepare all solutions using ultrapure water (conductivity lower than 0.05 μS/cm at 25 °C) and analytical grade reagents or better. Store all solutions at 4 °C and allow standing at room temperature before every use. Pass all solutions, excepting the sheath-liquid, through 0.22 μm nylon filters before CE-MS and C18-SPE-CE-MS. Degas for 10 min the BGE and the sheath liquid in an ultrasonic bath before the first use.

1. Neuropeptide standard solutions: Prepare 2500 μg/mL stock individual solutions of each peptide (Met, End, and DynA). Dissolve with the appropriate volume of water the whole

amount of solid in the vial provided by the manufacturer (*see* **Note 1**). Use the stock individual solutions to prepare 1 mL of a 100 μg/mL stock mixture solution of the three peptides. Store all stock solutions as 25 μL aliquots in plastic vials at −20 °C and thaw before use (*see* **Note 2**). Prepare diluted standard mixtures of the neuropeptides at different concentrations from the 100 μg/mL standard mixture (*see* **Note 3**).

2. Plasma samples: Collect human venous blood samples in standard plasma collection tubes. Place on ice. Separate plasma from blood cells by centrifugation (10 min at 4000 × *g*). Store 200 μL aliquots at −20 °C and thaw before use (*see* **Note 2**).
3. Separation or background electrolyte (BGE): 50 mM acetic acid (HAc)-50 mM formic acid (HFor), pH 3.5 (*see* **Note 4**). Add about 90 mL water to a glass beaker. Add with a micropipette the volumes of HAc and HFor to prepare 100 mL of BGE (around 290 and 190 μL, respectively). Mix and adjust pH with NH_3 (*see* **Note 5**). Transfer to a 100 mL volumetric flask and make up to the final volume with water. Transfer to a 125 mL glass bottle and check the pH value. If necessary, readjust the pH value with the smallest volume of a diluted NH_3 solution. Cap the bottle and store at 4 °C.
4. Eluent: 60:40 v/v methanol:water with 50 mM HAc-50 mM HFor (*see* **Note 6**). Add 60 mL of 2-propanol, 40 mL of water and the volumes of HAc and HFor to prepare 100 mL of eluent (around 290 and 190 μL, respectively). Cap, mix, and store at 4 °C.
5. Capillary activation solution: 1 M NaOH. Weigh 4 g of sodium hydroxide pellets in a 125 mL glass bottle. Dissolve with 100 mL of water. Cap and store at 4 °C (*see* **Note 7**).
6. Sheath liquid: 60:40 v/v 2-propanol:water with 0.5 % v/v HFor (*see* **Note 8**). Add 60 mL of 2-propanol, 40 mL of water and 50 μL of HFor to a 125 mL glass bottle. Cap, mix, and store at 4 °C.
7. Bare fused silica capillary: 75 μm internal diameter (id) × 360 μm outer diameter (od) (at least 1 m) and 250 id × 360 μm od (at least 5 cm).
8. Binocular stereomicroscope with a magnification range up to 100× (*see* **Note 9**).
9. Fused silica capillary column cutter with a rotating diamond blade (*see* **Note 10**).
10. C18 SPE cartridge (*see* **Note 11**).
11. Microspatula: 6.4 cm total length (L_T) and 100 μm of blade width (Cole-Parmer, Vernon Hills, IL, USA).
12. Peristaltic pump Tygon® plastic tube: 250 μm id (orange-blue retaining stops) (*see* **Note 12**).
13. Centrifugal filters of 10 K Mr cut-off (*see* **Note 13**).

3 Methods

3.1 Plasma Sample Pretreatment

1. Add 1200 µL of cold acetonitrile (4 °C) to 200 µL of plasma (*see* **Note 14**). Vortex for 30 s and keep at −20 °C for 1 h. Centrifuge the mixture (8 min at 700 × *g*) and collect the supernatant. Add 100 µL of acetonitrile to the solid residue. Centrifuge the mixture (8 min at 1200 × *g*). Pool the supernatant with the first one. Rinse with 100 µL of acetonitrile the micropipette tip used to deliver the supernatants. Mix the solvent with the supernatants.
2. Evaporate to dryness under a stream of compressed air the pooled extract (*see* **Note 15**). Reconstitute the solid residue with 100 µL of water.
3. The day before, passivate the centrifugal filters with a 5% v/v PEG (8 K, M_r) solution [37] (*see* **Note 13**).
4. Add the 100 µL aqueous plasma extract to the passivated reservoir and centrifuge (10 min at 11,000 × *g*). Wash twice the filtration residue with 50 µL of water (10 min at 11,000 × *g* and 8 min at 11,000 × *g*). Pool the three filtrates. Measure the final volume with a micropipette and add water until 200 µL (*see* **Note 14**).

3.2 SPE-CE-MS

Some of the capillary flushes have to be manually performed (*see* **Note 16**). In the other cases, work at room temperature with the capillaries in the commercial cartridge cassette for CE-MS and install it in your instrument (*see* **Note 17**).

3.2.1 Fused Silica Capillary Preparation

1. Activate a 72 cm total length (L_T) × 75 µm id × 360 µm od capillary to use as *separation capillary* (*see* **Note 18**). Flush capillary with 1 M NaOH (20 min), water (15 min) and BGE (15 min) (*see* **Note 7**). Equilibrate by applying the separation voltage (18 kV, normal polarity, cathode in the outlet) for 15 min. Cut it into two pieces of 7.5 and 64.5 cm (*see* **Note 19**). Flush manually with water (*see* **Note 16**).
2. Cut a 0.7 cm L_T × 250 µm id × 360 µm od capillary to use as *microcartridge body*. Flush manually with water (*see* **Note 16**).

3.2.2 Construction of a Double-Frit Microcartridge

The construction of a double-frit microcartridge is schematically described in Fig. 1 [8, 18, 30–34]**.** Do not forget to control all the steps with the microscope (*see* **Note 9**).

1. Connect a long piece of plastic tube to the 7.5 cm piece of separation capillary. Push the capillary until it is 0.25 cm inside the plastic tube. Cut the plastic tube with a scalpel to obtain a connector of 0.5 cm.
2. Use a scalpel to cut into small pieces (approx. 100 µm diameter, Fig. 1a) one of the original polyethylene filters of the SPE cartridge (*see* **Note 20**). Collect all the microfrits in a plastic

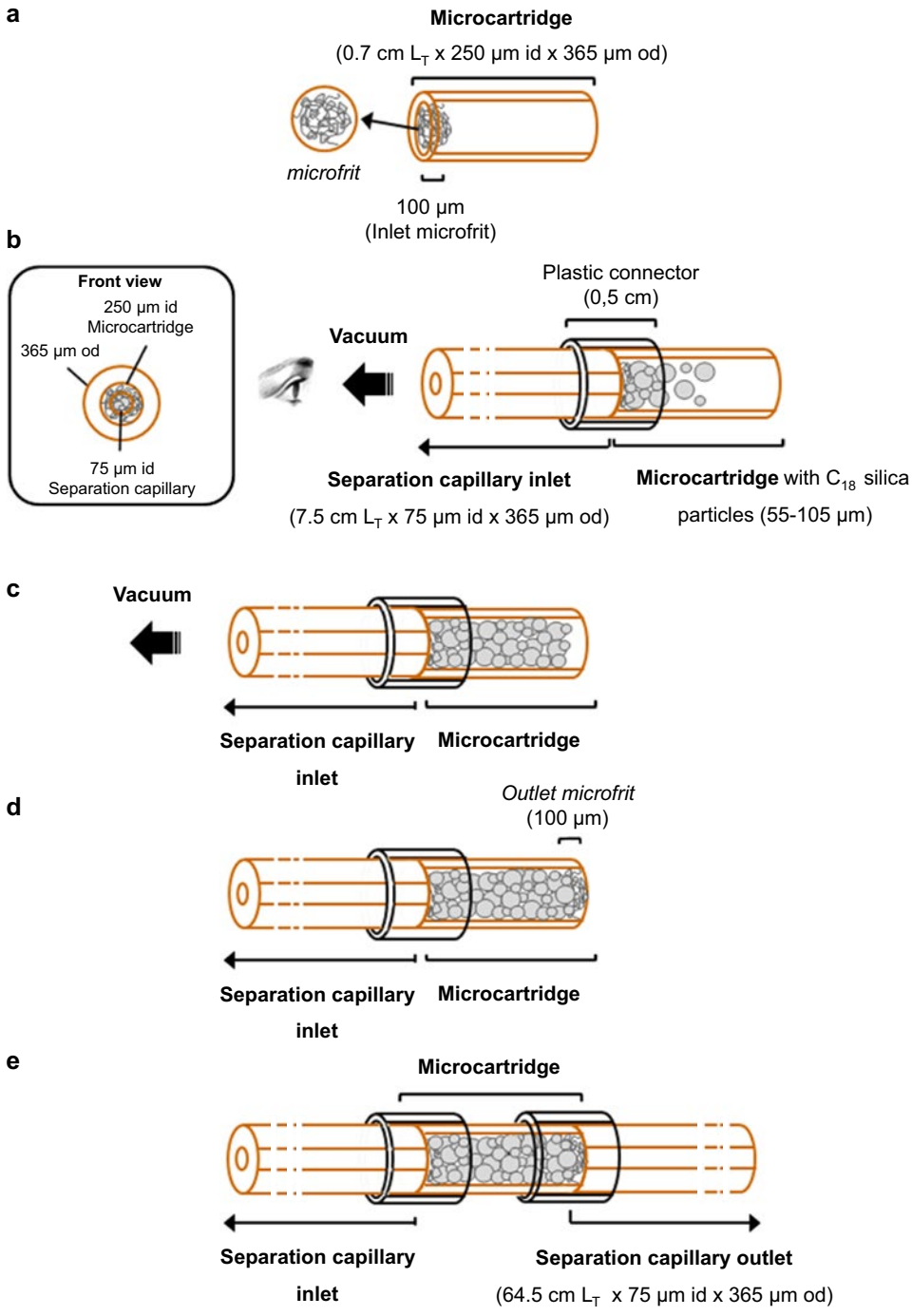

Fig. 1 Construction procedure of a double-frit SPE-CE microcartridge (or analyte concentrator)

vial. For ease of handing, connect the microcartridge body to the adapted needle (*see* **Note 16**). Flush manually with water. Use the microspatula to introduce a microfrit in the free end of the microcartridge body (inlet frit) (Fig. 1a).

3. Connect the inlet of the microcartridge body with the frit to the 7.5 cm piece of separation capillary with the plastic connector. Be careful not to lose the microfrit. Push carefully the microcartridge body inside the plastic connector until you do not observe dead volume between the microcartridge body and the 7.5 cm piece of separation capillary (*see* **Note 12**). Disconnect the microcartridge body from the plastic connector of the flat-tip needle (*see* **Note 12**).
4. Prepare a simple and reusable device to fill the microcartridge with sorbent particles by vacuum. Cut 1 cm from the bottom of the body of a 5 mL disposable polypropylene syringe. Fit it in an appropriate plastic vacuum hose (*see* **Note 21**). Connect the adapted needle to the syringe tip. Then, connect the free end of the 7.5 cm piece of separation capillary (inlet). Pack the microcartridge by vacuum with C18 sorbent particles until it is completely full (*see* **Note 22**). Use a soft paper towel, before turning off the vacuum, to eliminate the particles from the outside. Pull carefully out the separation capillary of the adapted needle (Fig. 1b).
5. Remove with the microspatula a 100 μm length of sorbent particles (Fig. 1c). Introduce the outlet microfrit pushing carefully with the microspatula (Fig. 1d).
6. Connect a long piece of plastic tube to one of the ends of the 64.5 cm piece of separation capillary. Push the capillary until it is 0.25 cm inside the plastic tube. Cut the plastic tube to obtain a connector of 0.5 cm.
7. Connect the outlet end of the microcartridge body to the 64.5 cm piece of separation capillary with the plastic connector to obtain the full-length capillary (*see* **Notes 12** and **23**). The microcartridge should appear as completely full under the microscope.
8. Flush manually the SPE-CE capillary with water to check the system for abnormal flow restriction. Discard it if water is not flowing.
9. Install the SPE-CE capillary inlet in the cartridge-cassette (*see* **Note 19**). Use a good adhesive tape to fix the separation capillary and the microcartridge positions.
10. Install the SPE-CE capillary outlet in the CE-MS sheath-flow interface. Be careful not to damage the separation capillary exit tip. Leave 10 cm of capillary outside the electrode tip. Screw the PEEK connector of the interface to fix capillary position. Burn with a lighter the polyimide of the outlet end and carefully remove the ashes with a soft paper towel soaked with ethanol (*see* **Note 24**). Unscrew the PEEK connector and fix the capillary outlet protrusion with regard to the electrode outlet to 0.1 cm (*see* **Note 25**). Set sheath liquid flow rate at 3.3 μL/min (*see* **Note 17**) and the MS parameters optimized for detection of the neuropeptides (*see* **Note 26**).

11. Flush the new SPE-CE capillary win BGE (10 min) and apply the separation voltage for 15 min (18 kV). Discard it if current is zero, much lower than expected or very unstable (drifts, spikes, etc.).

3.2.3 Preconcentration and Clean-Up

Optimum sensitivity, clean-up, and separation are basically obtained with an appropriate balance between the loading, washing and BGE solutions composition and step durations, the elution plug volume and composition and the separation voltage.

1. Flush the SPE-CE capillary win water (1 min), methanol (1 min), water (1 min), and BGE (3 min) for conditioning (*see* **Note 27**).
2. Flush with diluted peptide solution (10 min) to load the sample (*see* **Note 28**).
3. Flush with BGE (2 min) to wash out the unretained molecules and to fill and equilibrate the capillary before the electrophoretic separation (*see* **Note 29**).
4. Inject (10 s at 50 mbar) the eluent to release the retained peptides (*see* **Note 30**).
5. Apply (20 min) the separation voltage of 18 kV with a BGE vial in the inlet capillary end and the outlet capillary end properly installed in the sheath-flow CE-MS interface (Figs. 2 and 3, *see* **Notes 17** and **31**).
6. Flush with acetonitrile (2 min) to prevent carry-over between consecutive analyses.
7. Discard the SPE-CE capillary if you observe current instability and breakdowns or deterioration of the extraction performance (*see* **Note 32**).

4 Notes

1. Methionine enkephalin (Tyr-Gly-Gly-Phe-Met, 573.23 Da, Met), endomorphin1 (Tyr-Pro-Trp-Phe-NH_2, 610.29 Da, End), and Dynorphin A (1–7) (Tyr-Gly-Gly-Phe-Leu-Arg-Arg, 867.47 Da, DynA). Peptides are provided as small amounts of lyophilized powders and are difficult to weigh accurately.
2. Thaw the aliquots using a water bath. It is not recommended to freeze an aliquot that has been previously frozen and thawed.
3. The 100 μg/mL standard mixture can be stored at 4 °C during several months (no stability tests have been run). The diluted standard mixtures or treated plasma samples have to be immediately discarded after the analysis (*see* **Note 2**).

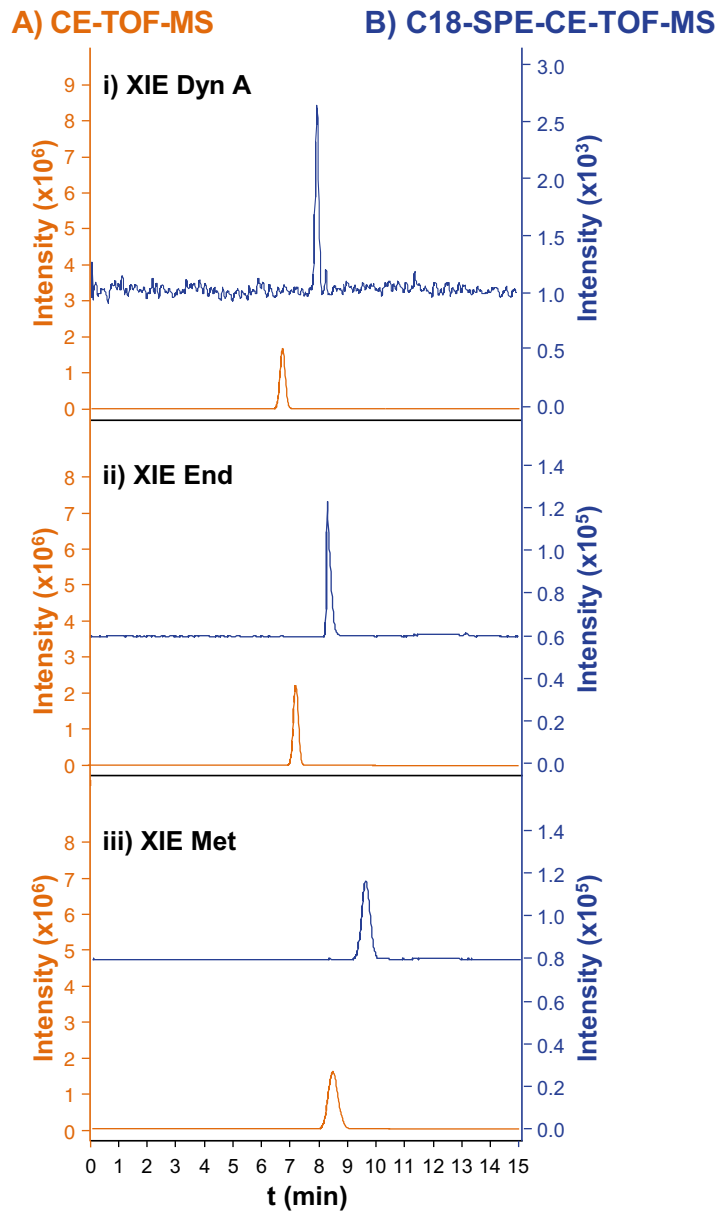

Fig. 2 Extracted ion electropherograms (XIE) of each neuropeptide for (**a**) a 10 μg/mL standard mixture by CE-MS (*orange*) and (**b**) a 1 ng/mL standard mixture by C18-SPE-CE-MS (*blue*). (i) DynA, (ii) End1 and (iii) Met

4. The pH value is optimized for separation of the standard mixture of neuropeptides. In this specific case, results are the same in terms of sensitivity enhancement with a BGE of 50 mM HAc-50 mM HFor (pH 2.3, without pH adjustment) or 1 M HAc (pH 2.1, without pH adjustment).

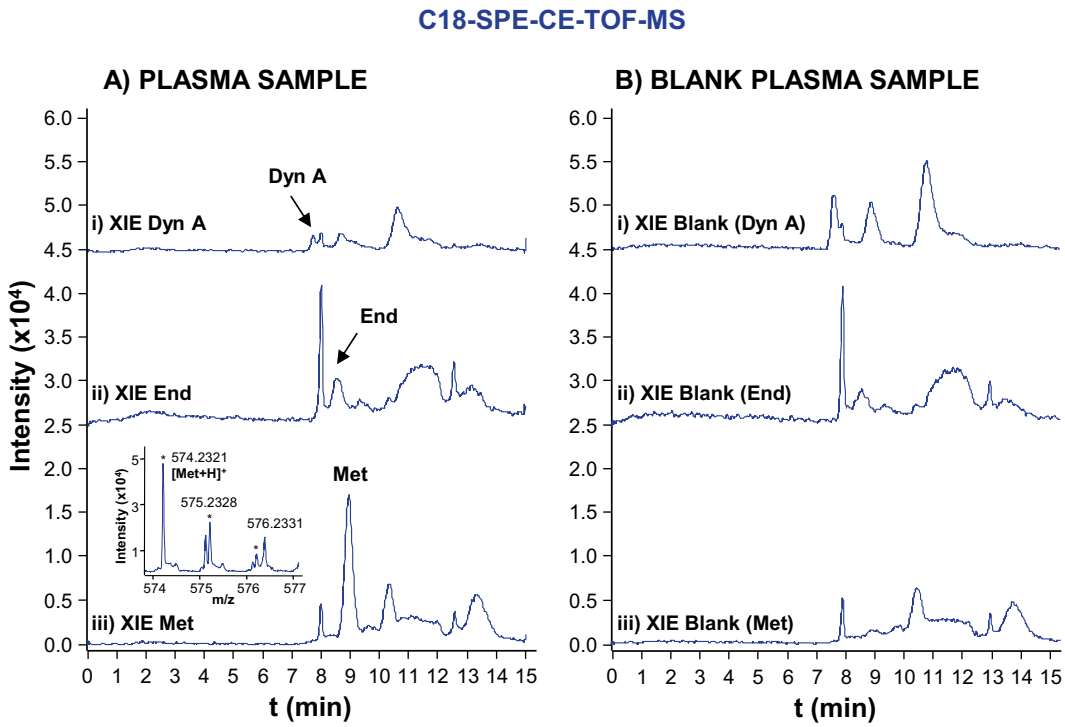

Fig. 3 XIE of each neuropeptide by C18-SPE-CE-MS for (**a**) a plasma sample spiked with 1 ng/mL of each neuropeptide and (**b**) a blank plasma sample. (i) DynA, (ii) End1, and (iii) Met (the mass spectrum of Met is shown as an example in the spiked plasma sample)

5. A concentrated NH_3 solution (25%) can be directly used to adjust the pH value (the volume to add is approximately 150 μL).
6. The eluent composition is optimized for preconcentration and separation of the standard mixture of neuropeptides. No pH adjustment is necessary.
7. Use 1 M NaOH solution to activate the fused silica capillary before assembling the microcartridge. Later, it will ruin the C18 sorbent in C18-SPE-CE-MS. The activation must be performed without installing the capillary outlet in the CE-MS sheath-flow interface to avoid the unnecessary entrance of NaOH into the mass spectrometer. This is a better option than turning off the nebulizer gas and the ESI voltage, while leaving the capillary outlet tip outside the metal electrode of the interface. In this last case, it is necessary to readjust the capillary protrusion in relation to the electrode outlet in the interface.
8. This sheath liquid composition provides, in combination with the indicated BGEs, sensitive and reproducible CE-MS analysis in positive ion mode for peptides and proteins and many other compounds.

9. Control all the operations involving capillaries with the stereomicroscope at 100× magnification (e.g. capillary cuts, capillary connexion dead volumes, polyimide removal from the capillary outlet for on-line MS detection or fabrication of the on-line SPE microcartridges).
10. A proper connexion between capillaries without dead volumes or a proper capillary outlet tip for CE-MS can be achieved if capillaries are cleanly cut. This type of tool is the best alternative to cut typical 360 μm od fused silica capillaries. It is available from different manufacturers.
11. A typical SPE cartridge (no matter the volume) can be easily disassembled pushing with an unfolded big paperclip (or similar) through the outlet cartridge hole. Conserve both the polyethylene filters (frits) and the C18 sorbent in plastic vials. The C18 sorbent from C18 Sep-pak® cartridges (Waters, Milford, MA, USA) provides excellent results in terms of preconcentration factors, reproducibility, and durability for these neuropeptides. Furthermore, the pore size and the diameter of the silica particles (i.e. 125 Å and 55–105 μm, respectively) are excellent to avoid flow restriction and bubble formation inside the microcartridges, precluding problems with sample introduction or current instability and breakdowns during the electrophoretic separation.
12. Connectors made from this tube provide a tight junction between two pieces of clean-cut 360 μm od fused silica capillaries. No adhesive sealing is necessary and the microcartridge is completely replaceable. To obtain a proper connexion without dead volumes between two capillary pieces, hold with one hand the shortest capillary piece and push the longest one against the connector. Nitrile gloves or a soft paper towel can be used to avoid capillary contamination during manipulation. Be careful not to twist or bend the capillaries during connexion. The 250 μm id fused silica capillary is especially fragile because the wall is very thin.
13. In Amicon® Ultra 0.5 filters of 10 K Mr cut-off (Millipore, Bedford, MA, USA), the cellulose-acetate membrane is in vertical position for tangential (or cross) flow filtration to prevent membrane fouling, due to solute polarization, and filtration to dryness. Passivation with a 5% v/v PEG (8 K, M_r) solution is necessary to avoid peptide loss through adsorption and limited peptide recoveries [37]. Add 500 μL of the passivation solution into the reservoir of the centrifugal device, cap, and allow standing 24 h at room temperature. Uncap and rinse thoroughly with tap water. Add 500 μL of (ultrapure) water and centrifuge to dead stop (30 min at 11,000 × *g*). Eliminate the remaining water by inverting the reservoir in the plastic vial and spinning at a reduced centrifugal force (2 min at 300 × *g*).

If available, because they have been discontinued for some time, you can use Microcon Amicon® YM-10 filters 10 K Mr cut-off (Millipore), in which the membrane is in horizontal position for direct filtration. With the old lots of these YM-10 filters passivation was not necessary [32].

14. To prepare fortified samples before the sample pretreatment, spike 180 μL of the plasma aliquot with a small volume (i.e. 20 μL) of a standard peptide mixture with an appropriate concentration to avoid excessive sample matrix dilution. To prepare fortified samples after the sample pretreatment, do the same before finishing making up volume with water to 200 μL. Blank samples are prepared spiking plasma with 20 μL of water before or after the sample pretreatment.
15. Use the typical SPE vacuum manifold with the drying attachment to evaporate the eluates. You can use the central lab compressed air system instead of nitrogen. Using appropriate gas flow rate, evaporation is gentle and takes around 2 h. Do not touch the solution surface with the needles and try to avoid splashes.
16. Use a 40 mm $L_T \times 0.8$ mm od hypodermic metal needle to prepare a needle adapted to fit 360 μm od capillaries. Sand the beveled tip of the hypodermic needle with small grit sandpaper. Wash the flat-tip needle with water. Connect a 0.5 cm L_T of the Tygon® plastic tube to the needle tip. Use the adapted needle in combination with a 5 mL disposable polypropylene syringe to flush manually 360 μm od capillaries (avoid syringes with rubber tips on plungers). While connecting a capillary, be careful not to push it too much inside the plastic connector to avoid contact between the flat-tip metal needle and the capillary. All capillaries need to be easily pulled out later. The 250 μm id capillary is especially fragile because of the very thin walls.
17. For ease of understanding, the SPE-CE-MS procedures are explained to be performed on a HP^{3D} CE instrument (Agilent technologies, Waldbronn, Germany) that allows flushing capillaries at 930 mbar and hydrodynamic injections at low pressures ranging from 0 to 50 mbar. The CE-MS interface is a sheath-flow interface (Agilent Technologies), and sheath liquid is delivered by a syringe pump KD Scientific 100 Series (Holliston, MA, USA) Nowadays, the syringe pumps typically used to do infusion experiments to calibrate the mass spectrometers provide the low flows with no pulsations necessary for a stable ESI signal. In general, the sheath liquid flows required in CE-MS with this interface are between 2 and 4 μL/min and separation capillary outlet is protruding from the electrode approximately 0.1 mm.
18. A 72 cm L_T separation capillary allows working comfortably taking into account the distance between the CE instrument

and the mass spectrometer entrance. The separation voltage is optimized for separation of this standard mixture of neuropeptides in a reasonable time. Furthermore, using this BGE, it allows electrophoretic currents lower than 50 μA, which are recommended to prevent electrical arcs between the electrode tip of the CE-MS interface and the mass spectrometer entrance.

19. The microcartridge will be at 7.5 cm from the inlet of the separation capillary. In Agilent Technologies CE instruments this is the best position near the inlet to fit it and no special modifications are necessary. The microcartridge is inside the cartridge-cassette which holds the full-length capillary in a coiled configured form**.** It is not necessary to use the UV detector, but in this instrument it is mandatory to install the UV alignment interface to allow the initialization of the control software.
20. Frits are a definitive solution to prevent microcartridge bleeding, but if they are too large or dense may promote column back pressure, electroosmotic (EOF) disturbance, bubble formation, and current drops or breakdowns. As an alternative to a double-frit microcartridge, a fritless microcartridge can be constructed with a 50 μm id separation capillary if the C18 sorbent particles are sieved with a 50 μm iron steel sieve. *See* **Notes 27** and **28** that explain the influence of separation capillary id on the sample volume loaded by pressure.
21. Use the central vacuum system of the laboratory or a compact vacuum pump as a vacuum source. The vacuum used with the typical chromatographic mobile phase filtration systems is enough.
22. The C18 sorbent particles are kept dry in a plastic vial. Do not use sorbent slurries. Use the extreme of the vacuum hose with the syringe as a handle to bury repeatedly the microcartridge body, with an upside down movement, in the C18 sorbent particles. If the microcartridge is not easily vacuum filled, the inlet microfrit is probably too large or dense. In this case, discard the microcartridge.
23. In our case, no adhesive sealing is necessary and the microcartridge is completely replaceable. In other cases, especially if microcartridge position or column installation inside the commercial cartridge cassette compromise microcartridge integrity, we recommend to cover the microcartridge and the connectors with a thin layer of epoxy resin to reinforce the construction before installation. Buy the epoxy resin in a local supermarket. Depending on the epoxy resin the cure time may vary from a few hours to more than 12 h.
24. The nonconductive polyimide coating is removed from the outlet end of the separation capillary to obtain an optimum mixture of the BGE with the sheath liquid and maintain a stable spray. Be careful to remove all ashes. Allow the sheath liquid to flow during 15 min before the first analysis.

25. The capillary must protrude enough from the metal electrode to allow the best sensitivity and reproducibility, with minimum mixing volume between the sheath liquid and the BGE.
26. The mass spectrometer set-up must be performed before beginning C18-SPE-CE-MS experiments. First, tune and calibrate the mass spectrometer using the standard solutions and following the instructions given by the manufacturer. Later, the parameters of the mass spectrometer can be automatically and finely tuned for the peptides of interest by direct infusion at 50 mbar of standard peptide solutions in BGE through a conventional CE-MS separation capillary. In this case we infuse a 10 μg/mL solution of Met and we evaluate the different MS parameters to maximize the signal for the singly charged molecular ion ($[Met+H]^+$). The nebulizer gas pressure, the drying gas flow rate and temperature have to be later directly optimized by CE-MS, because they depends on the analytes, the BGE and sheath liquid composition and flow rates, and the separation and ionization voltages. In this case are set at 7 psi (nebulizer gas pressure), 4 L/min (drying gas flow rate), and 200 °C (drying gas T), respectively.
27. The duration of the flushes, loading, and injection steps at a certain pressure are optimized for this particular case. In order to avoid the effects of the nebulizer gas or the ESI voltage during flushes, loading or injection steps both can be switched off until applying the voltage for the electrophoretic separation. It is preferable to introduce the sample hydrodynamically, to avoid selectivity of electrokinetic injection. In general, as there is no backpressure due to the microcartridge, the volumes in hydrodynamic introduction can be estimated using the Hagen–Poiseuille equation:

$$\frac{\Delta P \times \mathrm{id}^4 \times \pi \times \mathrm{t}}{128 \times \eta \times \mathrm{L}_T}$$

where ΔP is pressure difference across the capillary, id is separation capillary internal diameter, t is introduction time, η is solution viscosity, and L_T is total separation capillary length. Backpressure can be checked flushing the SPE-CE capillary with water, measuring the time required to collect in a plastic vial a certain volume (e.g. 1 μL, use a micropipette for the measurement) and comparing result with a conventional capillary with the same dimensions. In general, the blockage promoting backpressure is due to large or dense frits or excessive sorbent packing. Discard the capillary if backpressure is high.
28. Wide id capillaries (i.e. 75 μm id) and high loading pressures (i.e. 930 mbar) minimize time necessary to load a certain volume of sample (approximately 60 μL, loading 10 min in a 72 cm L_T capillary) (*see* **Note 27**). A loading time of 10 min at

930 mbar is a good compromise between volume of sample loaded, total analysis time, limit of detection, linearity, reproducibility, and microcartridge durability. With this C18 sorbent sensitivity enhancement is not improved loading the same sample volume at 50 mbar. Loading 60 μL at 50 mbar required a longer time than at 930 mbar and probably promoted analyte breakthrough (the amount of eluted analyte overcomes the amount of retained analyte during the loading step) [8].

In this approach, the sensitivity enhancement with extremely diluted samples is always limited by analyte breakthrough during sample loading. If desired, with other peptides or compounds, the sample loading time can be further optimized. Analyze standard peptide mixtures of different concentrations loading for 10 min at 930 mbar until arriving to a concentration close to the LOD. Analyze this solution extending the loading times until 30 (approx. 180 μL), 45 (approx. 270 μL), and 60 min (approx. 360 μL) (*see* **Note 27**). Represent a graph of eluted peptide peak area versus loading time. Estimate the time to arrive to the breakthrough volume (peak area does not change or decrease). Select the longest loading time before analyte breakthrough starts. Check if it is possible to detect further diluted samples under the new conditions.

Loading a volume of sample larger than 360 μL in a reasonable time in the Agilent Technologies is hindered by the limited maximum pressure provided by the CE instrument (60 min at 930 mbar). Check a higher loading pressure if possible in your instrument. Another alternative could be to use other microcartridge design that allow a faster loading by pressure such as the cruciform and staggered designs proposed by N.A. Guzman [9, 10]**.** In such designs sample loading time by pressure is not determined by the separation capillary dimensions, because loading direction is orthogonal to the separation capillary. Another advantage is that sample loaded does not contact with the inner surface of the separation capillary, preventing sample matrix adsorption in the inner capillary wall.

29. Be aware that long or harsh washing steps to remove interferences may also promote analyte elution, hence lower preconcentration factors.

30. The injection volume (10 s at 50 mbar, approx. 50 nL, *see* **Note 27**) is optimized for quantitative elution and good separation of the standard mixture of neuropeptides.

31. Following this method, Met, End, and Dyn A can be detected until 0.1 ng/mL in standard mixtures by C18-SPE-CE-MS, which meant a 5000-fold improvement of the LODs compared to CE-MS (0.5 μg/mL) (Fig. 2). In spiked plasma samples the three peptides can be detected until 1 ng/mL due to the complexity of the plasma matrix and the limited selectivity of the C18 sorbent. With complex samples it is important to

use a selective detection, such as MS detection, that allows a reliable identification of the different peptides (Fig. 3). Further sensitivity enhancement can be obtained with C18 sorbents with minor changes on this method, combining transient isotachophoresis [18] or a sheathless CE-MS nanoelectrospray interface [36].

32. A SPE-CE capillary can be used up to around 20 runs with standards and up to around 10 runs with pretreated plasma samples. To replace the microcartridge, uninstall the capillary from the cartridge-cassette and the CE-MS interface and disconnect the microcartridge from both pieces of separation capillary. Discard the microcartridge. Discard both pieces of separation capillary if the tips in contact with the microcartridge are damaged. Alternatively, you can discard the inlet piece and cut a very short length (0.2–0.3 cm) of the outlet piece. Prepare a new inlet piece of separation capillary. Construct a new SPE-CE capillary.

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References

1. Kostal V, Katzenmeyer J, Arriaga EA (2008) Capillary electrophoresis in bioanalysis. Anal Chem 80:4533–4550
2. Kasicka V (2010) Recent advances in CE and CEC of peptides (2007-2009). Electrophoresis 31:122–146
3. Kasicka V (2012) Recent developments in CE and CEC of peptides (2009-2011). Electrophoresis 33:48–73
4. Kasicka V (2014) Recent developments in capillary and microchip electroseparations of peptides (2011-2013). Electrophoresis 35:69–95
5. Simpson SL, Quirino JP, Terabe S (2008) On-line sample preconcentration in capillary electrophoresis. Fundamentals and applications. J Chromatogr A 1184:504–541
6. Breadmore MC, Dawod M, Quirino JP (2011) Recent advances in enhancing the sensitivity of electrophoresis and electrochromatography in capillaries and microchips (2008-2010). Electrophoresis 32:127–148
7. Breadmore MC, Tubaon RM, Shallan AI, Phung SC, Keyon ASA, Gstoettenmayr D, Prapatpong P, Alhusban AA, Ranjbar L, See HH, Dawod M, Quirino JP (2015) Recent advances in enhancing the sensitivity of electrophoresis and electrochromatography in capillaries and microchips (2012–2014). Electrophoresis 36:36–61
8. Benavente F, Vescina MC, Hernández E, Sanz-Nebot V, Barbosa J, Guzman NA (2007) Lowering the concentration limits of detection by on-line solid-phase extraction-capillary electrophoresis-electrospray mass spectrometry. J Chromatogr A 1140:205–212
9. Guzman NA, Blanc T, Phillips TM (2008) Immunoaffinity capillary electrophoresis as a powerful strategy for the quantification of low-abundance biomarkers, drugs, and metabolites in biological matrices. Electrophoresis 29: 3259–3278
10. Guzman NA, Phillips TM (2011) Immunoaffinity capillary electrophoresis: a new versatile tool for determining protein biomarkers in inflammatory processes. Electrophoresis 32:1565–1578
11. Ramautar R, de Jong GJ, Somsen GW (2012) Developments in coupled solid-phase extraction-capillary electrophoresis 2009-2011. Electrophoresis 33:243–250

12. Ramautar R, Somsen GW, de Jong GJ (2014) Developments in coupled solid-phase extraction-capillary electrophoresis 2011-2013. Electrophoresis 35:128–137
13. Xu L, Dong X-Y, Sun Y (2009) Novel negatively charged tentacle-type polymer coating for on-line preconcentration of proteins in CE. Electrophoresis 30:689–695
14. Busnel J-M, Lion N, Girault HH (2008) Electrokinetic supercharging for highly efficient peptide preconcentration in capillary zone electrophoresis. Electrophoresis 29:1565–1572
15. Chen Y, Zhang L, Cai Z, Chen G (2011) Dynamic pH junction-sweeping for on-line focusing of dipeptides in capillary electrophoresis with laser-induced fluorescence detection. Analyst 136:1852–1858
16. Wang C-W, Her G-R (2014) The development of a counterflow-assisted preconcentration technique in capillary electrophoresis electrospray-ionization mass spectrometry. Electrophoresis 35:1251–1258
17. Xia S, Zhang L, Qiu B, Lu M, Chi Y, Chen G (2008) On-line preconcentration and quantitative analysis of peptide hormone of brain and intestine using on-column transient isotachophoresis coupled with capillary electrophoresis/electrospray ionization mass spectrometry. Rapid Commun Mass Spectrom 22:3719–3726
18. Medina-Casanellas S, Benavente F, Barbosa J, Sanz-Nebot V (2011) Transient isotachophoresis in on-line solid phase extraction capillary electrophoresis time-of-flight-mass spectrometry for peptide analysis in human plasma. Electrophoresis 32:1750–1759
19. Wang Y, Fonslow BR, Wong CCL, Nakorchevsky A, Yates JR (2012) Improving the comprehensiveness and sensitivity of sheathless capillary electrophoresis-tandem mass spectrometry for proteomic analysis. Anal Chem 84:8505–8513
20. Heemskerk AAM, Wuhrer M, Busnel J-M, Koeleman CAM, Selman MHJ, Vidarsson G, Kapur R, Schoenmaker B, Derks RJE, Deelder AM, Mayboroda OA (2013) Coupling porous sheathless interface MS with transient-ITP in neutral capillaries for improved sensitivity in glycopeptide analysis. Electrophoresis 34:383–387
21. Heemskerk AAM, Deelder AM, Mayboroda OA (2016) CE-ESI-MS for bottom-up proteomics: advances in separation, interfacing and applications. Mass Spectrom Rev 35: 259–271
22. Gahoual R, Busnel J-M, Beck A, François Y-N, Leize-Wagner E (2014) Full antibody primary structure and microvariant characterization in a single injection using transient isotachophoresis and sheathless capillary electrophoresis-tandem mass spectrometry. Anal Chem 86: 9074–9081
23. Su S, Yu Y (2009) Online preconcentration of recombinant Arg-Gly-Asp-hirudin using dynamic pH junction for analysis in human urine samples by capillary electrophoresis-mass spectrometry. J Chromatogr A 1216: 1490–1495
24. Ye H, Xia S, Lin W, Yu L, Xu X, Zheng C, Liu X, Chen G (2010) CE-ESI-MS coupled with dynamic pH junction online concentration for analysis of peptides in human urine samples. Electrophoresis 31:3400–3406
25. Zhu G, Sun L, Yan X, Dovichi NJ (2014) Bottom-up proteomics of Escherichia coli using dynamic pH junction preconcentration and capillary zone electrophoresis-electrospray ionization-tandem mass spectrometry. Anal Chem 86:6331–6336
26. Ballard JNM, Lajoie GA, Yeung KK-C (2007) Selective sampling of multiply phosphorylated peptides by capillary electrophoresis for electrospray ionization mass spectrometry analysis. J Chromatogr A 1156:101–110
27. Sun L, Hebert AS, Yan X, Zhao Y, Westphall MS, Rush MJP, Zhu G, Champion MM, Coon JJ, Dovichi NJ (2014) Over 10000 peptide identifications from the HeLa proteome by using single-shot capillary zone electrophoresis combined with tandem mass spectrometry. Angew Chem Int Ed Engl 53:13931–13933
28. Krenkova J, Foret F (2012) On-line CE/ESI/MS interfacing: recent developments and applications in proteomics. Proteomics 12: 2978–2990
29. Bonvin G, Schappler J, Rudaz S (2012) Capillary electrophoresis-electrospray ionization-mass spectrometry interfaces: fundamental concepts and technical developments. J Chromatogr A 1267:17–31
30. Hernández E, Benavente F, Sanz-Nebot V, Barbosa J (2007) Analysis of opioid peptides by on-line SPE-CE-ESI-MS. Electrophoresis 28:3957–3965
31. Hernández E, Benavente F, Sanz-Nebot V, Barbosa J (2008) Evaluation of on-line solid phase extraction-capillary electrophoresis-electrospray-mass spectrometry for the analysis of neuropeptides in human plasma. Electrophoresis 29:3366–3376
32. Benavente F, Medina-Casanellas S, Barbosa J, Sanz-Nebot V (2010) Investigation of commercial sorbents for the analysis of opioid peptides in human plasma by on-line SPE-CE. J Sep Sci 33:1294–1304
33. Medina-Casanellas S, Benavente F, Barbosa J, Sanz-Nebot V (2012) Preparation and evaluation of an immunoaffinity sorbent for the analysis

of opioid peptides by on-line immunoaffinity solid-phase extraction capillary electrophoresis-mass spectrometry. Anal Chim Acta 717: 134–142

34. Medina-Casanellas S, Benavente F, Barbosa J, Sanz-Nebot V (2013) Preparation and evaluation of an immunoaffinity sorbent with Fab' antibody fragments for the analysis of opioid peptides by on-line immunoaffinity solid-phase extraction capillary electrophoresis-mass spectrometry. Anal Chim Acta 789:91–99
35. Ortiz-Villanueva E, Benavente F, Giménez E, Yilmaz F, Sanz-Nebot V (2014) Preparation and evaluation of open tubular C18-silica monolithic microcartridges for preconcentration of peptides by on-line solid phase extraction capillary electrophoresis. Anal Chim Acta 846:51–59
36. Medina-Casanellas S, Domínguez-Vega E, Benavente F, Sanz-Nebot V, Somsen GW, de Jong GJ (2014) Low-picomolar analysis of peptides by on-line coupling of fritless solid-phase extraction to sheathless capillary electrophoresis-mass spectrometry. J Chromatogr A 1328:1–6
37. Pont L, Benavente F, Barbosa J, Sanz-Nebot V (2013) An update for human blood plasma pretreatment for optimized recovery of low-molecular-mass peptides prior to CE–MS and SPE–CE–MS. J Sep Sci 36:3896–3902

Chapter 7

Affinity Monolith-Integrated Microchips for Protein Purification and Concentration

Changlu Gao, Xiuhua Sun, Huaixin Wang, Wei Qiao, and Bo Hu

Abstract

Affinity chromatography is a valuable method to purify and concentrate minute amount of proteins. Monoliths with epoxy groups for affinity immobilization were prepared by direct in-situ photopolymerization of glycidyl methacrylate and ethylene glycol dimethacrylate in porogenic solvents consisting of 1-dodecanol and cyclohexanol. By integrating affinity monoliths onto a microfluidic system, targeted biomolecules can be captured and retained on affinity column, while other biomolecules having no specific interactions toward the immobilized ligands flow through the microchannel. Therefore, proteins which remain on the affinity column are purified and concentrated, and then eluted by appropriate solutions and finally, separated by microchip capillary electrophoresis. This integrated microfluidic device has been applied to the purification and separation of specific proteins (FITC-labeled human serum albumin and IgG) in a mixture.

Key words Affinity, Monolith, Microchip, Purification, Concentration

1 Introduction

Affinity chromatography is a separation technique based upon the specific and reversible binding of a protein to a matrix-bound ligand. This highly specific interaction of ligand and biomolecules can be utilized to purify targeted proteins, while other ingredients are unretained on the chromatographic column [1, 2]. Affinity chromatography has been widely used in sample pretreatment. But the sample loss can be tremendous during purification of small amounts of biological samples. Therefore, a microchip capillary electrophoresis (CE) has been developed for the analysis of biological samples at a miniaturized scale [3]. A major benefit of miniaturization is a reduction of both sample and reagent consumption, which make the assay of minute amount of biological samples more attractive. To widen the applicability and enhance the performance of CE microchips, various sample pretreatment techniques, including concentration and dilution [4, 5], purification and filtering [6], dialysis [7, 8], have been demonstrated. For the purification of proteins, a chromatographic matrix with high capacity for ligand

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immobilization and compatibility for microchip CE is needed. Currently, monoliths are good candidates as stationary-phase chromatographic media compared with packed beads by virtue of their porous structures and ease of in-situ fabrication. Monolithic polymers with large interconnected pores may be prepared in a simple way from a homogenous mixture and located into a defined position of channels allowing for high flow rates at moderate pressure. By changing the monomer or cross-linker, different monoliths with large surface areas and functional groups can be obtained [9, 10]. Recently, functionalized photopolymerized monoliths have been used for sample preparation in chemical [11], DNA [12], and protein analysis [13, 14]. Combining monolithic column bearing reactive groups with microchip techniques can concentrate/purify and separate proteins to achieve various purposes. In our work, glycidyl methacrylate-based monoliths were fabricated inside the microfluidic channel, anti-FITC antibodies were immobilized on the monoliths for capturing FITC-labeled proteins, by selecting appropriate elution conditions, the monolith-concentrated proteins were then eluted and separated.

2 Materials

Prepare all solutions using ultrapure water (obtained by purifying deionized water to attain a resistance of 18 M Ω at 25 °C) and analytical grade reagents. Prepare and store all reagents at room temperature (unless indicated otherwise). Diligently follow all waste disposal regulations when disposing waste materials. Do not add sodium azide to the reagents.

2.1 Microchip Fabrication

1. Silicon wafers <100> (TTI Silicon).
2. CleWin software (WieWeb Software).
3. 5080 dpi transparency films.
4. 2 mm thick poly(methyl methacrylate) (PMMA) (Acrylite FF).
5. AZ 4600 photoresist and its developer.
6. Hydrogen fluoride (47%) (HF), stored at 4 °C.
7. Potassium hydroxide.

2.2 Monolith Fabrication

1. Prepolymer solution: mix 0.3 g of glycidyl methacrylate (GMA, 97%), 0.2 g of ethylene glycol dimethacrylate (EGDMA, 98%), 0.35 g of 1-dodecanol (98%), 0.15 g of cyclohexanol (>99%), and 0.025 g of 2,2-dimethoxy-2-phenylacetophenone (DMPA, 98%) in a 2 ml glass, bubble with nitrogen gas for 3 min before sonication (*see* **Note 1**).
2. Cleaning buffer for monoliths: isopropanol, double distilled water.

3. Rinsing buffer for monoliths: 10 mM sodium dihydrogenphosphate and disodium hydrogenphosphate pH 7.2, 0.5% (w/w) hydroxypropyl cellulose (MW 806.9).

2.3 Antibody Immobilization Components

1. Goat anti-FITC was from Biomeda. Store at 4 °C.
2. Hydroxypropyl cellulose (HPC) (MW 806.9).
3. Antibody immobilization buffer: 0.05 M sodium borate and boric acid, pH 8.0. Store at 4 °C.
4. Blocking buffer: 0.1 M Tris–HCl, pH 8. Store at 4 °C.
5. Rinsing buffer: 10 mM sodium dihydrogenphosphate and disodium hydrogenphosphate pH 7.2, 0.5% hydroxypropyl cellulose. Store at 4 °C.

2.4 Protein Purification/ Separation

1. FITC-labeled immunoglobulin G (fluorescein isothiocyanate IgG) from human serum. Store at 4 °C.
2. FITC-labeled human serum albumin (fluorescein isothiocyanate HSA). Store at 4 °C.
3. Elution buffer: 200 mM acetic acid in water. Store at 4 °C.
4. Separation buffer: 10 mM sodium dihydrogenphosphate and disodiumhydrogenphosphate, 0.5% hydroxypropyl cellulose, pH 7.2. Store at 4 °C.
5. Sample: mixture of 20 μg/mL FITC-HSA and 50 μg/mL FITC-IgG in water. Store at 4 °C.

3 Methods

Carry out all procedures at room temperature unless otherwise specified.

3.1 Silicon Template Fabrication

1. Grow a thickness of 0.8 μm layer of silicon dioxide on a 3-in.-diameter silicon wafer. <100> at 1110 °C using a tube furnace in an atmosphere of oxygen (*see* **Note 2**).
2. Spin a layer of AZ 4600 photoresist on the wafer at ~7600 × *g*, the time for spinning is 90 s.
3. Bake the photoresist at 100 °C for 1 min on a hot plate (*see* **Note 3**).
4. Draw the pattern with CleWin software and print it onto transparency film with a 5080 dpi resolution as photomask.
5. Expose the photoresist under an Hg arc lamp at 30 mW/cm^2 for 30 s through the photomask.
6. Develop the photoresist in AZ developer solution.
7. Post-bake the developed photoresist at 120 °C in a convection oven for 15 min to harden the photoresist (*see* **Note 4**).

8. Etch the silicon wafer in 10% buffered HF solution (HF: $NH_4F = 1:6$) to remove the silicon dioxide layer from the areas no longer protected by photoresist (*see* **Note 5**).
9. Etch the wafer in 40% aqueous KOH solution for 35 min at 70 °C (*see* **Note 6**).

3.2 Poly(Methyl Methacrylate) Microchip Fabrication

1. Cut 4.8 cm × 2 cm × 0.4 cm poly(methyl methacrylate) (PMMA) pieces by CO_2 laser cutter.
2. Clean the PMMA pieces with soapy water, and dry them with air.
3. Emboss the elevated features in the etched Si template into PMMA substrates in a convection oven at 140 °C for 30 min to form the 100 μm wide, 10 μm deep channels (*see* **Note 7**).
4. Assemble the patterned PMMA sheet with an unimprinted PMMA piece by clamping the substrates together with two C-clamps, and place the assembly in the 110 °C convection oven for 30 min to get a bonded microchip device (*see* **Note 8**).

3.3 Photopatterning of Monoliths in Microchips

1. Prepolymer solution was made by mixing 0.3 g of GMA as functional monomer, 0.2 g of EGDMA as crosslinker, 0.35 g of 1-dodecanol and 0.15 g of cyclohexanol as porogens, and 0.025 g of DMPA as initiator under sonication for 5 min followed by bubbling nitrogen gas for 10 min before loading into a device (*see* **Note 9**).
2. Transfer a few drops of the prepolymer solution into reservoir 2 until it fills the targeted location (*see* **Note 10**).
3. Use an aluminum foil with a 5 mm × 2 mm opening as mask to define the 2 mm long monolith inside the channel (Fig. 1) (*see* **Note 11**).
4. Place the microchip under a 400 W UV Lamp for 5 min (*see* **Note 12**).
5. Remove the unreacted monomer and porogen by flushing isopropanol through the microchannels using a syringe pump after polymerization (*see* **Note 13**).
6. Rinse the monoliths and microchannels with rinsing buffer for future use, the end-on image of monoliths inside microfluidic channels are shown in Fig. 2. Fabricated monoliths are stored at 4 °C (*see* **Note 14**).

3.4 Immobilization of Anti-FITC

1. Add a drop of 1 mg/mL anti-FITC in antibody immobilization buffer in reservoir 2 to fill the monolithic channel (*see* **Note 15**).
2. Seal all the reservoirs with 3 M Scotch tape to prevent evaporation of solutions and put on a horizontal vibration shaker (reciprocating vibration, 80 rounds/min) at 37 °C for 24 h.

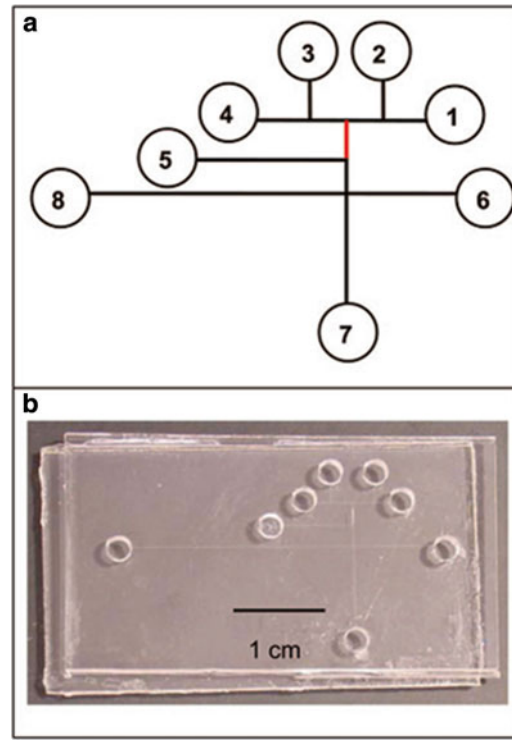

Fig. 1 Microchip layout. (**a**) Schematic design; reservoirs are (1) rinse, (2) protein standard, (3) sample, (4) elution solution, (5) waste, (6) buffer, (7) injection reservoir, and (8) high voltage power supply. The monolith location is indicated by the *red line*. (**b**) Photograph of a fabricated microchip. (Reproduced from ref. [13] with permission from ACS publications)

3. Flow blocking buffer through the monolith by syringe pump with a velocity of 1 μL/min by syringe pump for 1 h to block remaining epoxy groups (*see* **Note 16**).
4. Flush all the channels with rinsing buffer.

3.5 Protein Purification/ Separation

1. Load the monolith with sample containing 20 μg/mL FITC-HSA and 50 μg/mL FITC-IgG by applying 600 V to reservoir 5 (separation buffer) while grounding reservoir 3 (sample) for 30 s, and apply floating voltages to other reservoirs (*see* **Note 17**).
2. Rinse the unbound proteins with separation buffer for 30 s by applying 600 V to reservoir 5 while grounding reservoir 1 (separation buffer), and apply floating voltages to other reservoirs (*see* **Note 18**).
3. Elute the retained proteins with elution buffer by applying 600 V for 1 min to reservoir 7 (separation buffer) while grounding reservoir 4 (elution buffer), and apply floating voltages to other reservoirs (*see* **Note 19**).

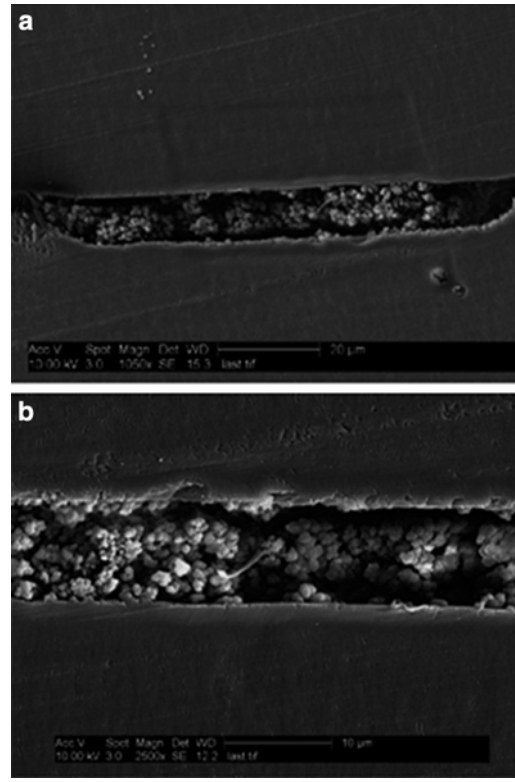

Fig. 2 End-on SEM images of monoliths inside a microfluidic channel. (**a**) Whole channel image; (**b**) magnified view. (Reproduced from ref. [13] with permission from ACS publications)

4. Separate the retained FITC-HSA by applying a 1400 V to reservoir 8 while maintaining reservoirs 4 and 7 at 600 V and grounding reservoir 6 (separation buffer), and apply floating voltages to other reservoirs (Fig. 3).

4 Notes

1. The mixture should be made right before the UV photopolymerization experiments.
2. The coating of SiO_2 can protect silicon from the etching by KOH.
3. This procedure can drive off residual solvents and improve adhesion of the photoresist to the wafer surface.
4. The convection oven can provide a more uniform temperature compared with hotplate, which helps the photoresist to adhere onto the wafer surface.

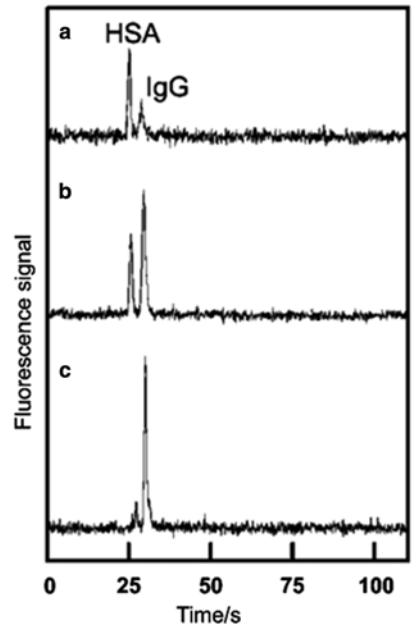

Fig. 3 Analyte-dependent monolith elution. (**a**–**c**) Three successive monolith elutions followed by microchip CE of FITC-HSA and FITC-IgG. Focus laser on the channel connected to reservoir 8, and collect fluorescence signal on a Nikon TE 300 inverted microscope through a Hamamatsu photomultiplier tube. (Reproduced from ref. [13] with permission from ACS publications)

5. Goggles, teflon apron are necessary to protect from corrosion by HF.
6. KOH is highly corrosive at high temperature, goggles and aprons are required.
7. The embossing process should be performed with extra care, because the uneven surfaces of silicon are extremely fragile during pressing.
8. The assembly can be disassembled after 20 min cooling at room temperature.
9. Keep the prepolymer solution away from heat and light, freshly made every day.
10. The solution can fill the whole channels by capillary force without pumping.
11. Aluminum foils are used as shield to prevent monolith polymerization in all the channels.
12. The appropriate UV amount to polymerize the monolith depends on the UV intensity and exposure time. A time duration of 15 min under a 20 mW/cm^2 UV lamp was used.

13. GMA, 1-dodecanol, and cyclohexanol are well dissolved in isopropanol, and isopropanol is amenable to PMMA.
14. The SEM images of GMA-based monoliths should be compact, porous, and uniform.
15. Buffer solution added on one of the reservoirs can travel through all the channels via capillary forces.
16. The amine group of Tris molecules can react with the remaining epoxy groups on monoliths.
17. The mixture of proteins was left to bind to the immunoaffinity monolith statically for 30 min to ensure adequate time for antigen–antibody interaction.
18. Until no fluorescence signal can be detected.
19. Shorter elution/injection times leads to insufficient fluorescence signal, while longer times provide better signal in the electropherograms, but leads to broader bands. As a compromise between signal and resolution, we used a 1 min elution/injection time.

References

1. Vickova M, Stettler A, Schwarz M (2006) Microchip affinity capillary electrophoresis: applications and recent advances. J Liq Chromatogr Relat Technol 29:1047–1076
2. Schou C, Heegaard NHH (2006) Recent applications of affinity interactions in capillary electrophoresis. Electrophoresis 27:44–59
3. Yang W, Sun X, Wang H et al (2009) Integrated microfluidic device for serum biomarker quantitation using either standard addition or a calibration curve. Anal Chem 81:8230–8235
4. Gong M, Wehmeyer KR, Limbach PA et al (2006) On-line sample preconcentration using field-amplified stacking injection in microchip capillary electrophoresis. Anal Chem 78: 3730–3737
5. Song S, Singh AK, Shepodd TJ et al (2004) Microchip dialysis of proteins using in situ photopatterned nanoporous polymer membranes. Anal Chem 76:2367–2373
6. Wolfe KA, Breadmore MC, Ferrance JP et al (2002) Toward a microchip-based solid-phase extraction method for isolation of nucleic acids. Electrophoresis 23:727–733
7. Huynh BH, Fogarty BA, Martin RS et al (2004) On-Line coupling of microdialysis sampling with microchip-based capillary electrophoresis. Anal Chem 76:6440–6447
8. Roddy ES, Price M, Ewing AG (2003) Continuous monitoring of a restriction enzyme digest of DNA on a microchip with automated capillary sample introduction. Anal Chem 75:3704–3711
9. Currivan S, Jandera P (2014) Post-polymerization modifications of polymeric monolithic columns. Chromatography 1:24–53
10. Trauner A, Mark HB, Williams HD (2011) Isolation of bacterial ribosomes with monolith chromatography. PLoS One 4:e16273
11. Yu C, Davey MH, Svec F et al (2001) Monolithic porous polymer for on-chip solid-phase extraction and preconcentration prepared by photoinitiated in situ polymerization within a microfluidic device. Anal Chem 73:5088–5096
12. Breadmore MC, Wolfe KA, Arcibal IG et al (2003) Microchip-based purification of DNA from biological samples. Anal Chem 75: 1880–1886
13. Sun X, Yang W, Pan T et al (2008) Affinity monolith-integrated poly(methyl methacrylate) microchips for on-line protein extraction and capillary electrophoresis. Anal Chem 80:5126–5130
14. Hahn R, Podgornik A, Merhar M et al (2001) Affinity monoliths generated by in situ polymerization of the ligand. Anal Chem 73:5126–5132

Chapter 8

Analysis of Somatropin by Double-Injection Capillary-Zone Electrophoresis in Polybrene/Chondroitin Sulfate A Double-Coated Capillaries

Ahmad Amini

Abstract

Purity determination of somatropin as a recombinant protein is important to ensure its safety and quality. This is carried out by capillary zone electrophoresis in double-injection mode using polybrene/chondroitin sulfate A double-coated capillaries. Modification of the capillary wall eliminates protein-wall interactions which results in improved accuracy and precision of the determinations. In the double-injection mode two somatropin samples are analyzed within a single electrophoretic run. Prior to the second injection, the first injected plug is electrophoresed for a predetermined time period in order to adjust the interplug distance. Here, the principle for the separation of somatropin charge variants is described.

Key words Double-injection capillary-zone electrophoresis (DICZE), Somatropin, Polybrene/chondroitin sulfate A (PB/CA) double-coated capillary, Partial electrophoresis time period (t_{PE})

1 Introduction

Recombinant human growth hormone (somatropin) being used in both the adult and pediatric populations is important for the growth of muscles and bones. Somatropin stimulates metabolism (of protein, carbohydrate, lipid, and mineral), tissue growth, as well as linear growth (height). The availability of somatropin has revolutionized the treatment of short stature resulting from GH deficiency, Turner syndrome, Noonan syndrome, Prader-Willi syndrome, short stature homeobox-containing gene (SHOX) deficiency, chronic renal insufficiency, idiopathic short stature, and children small for gestational age [1].

Capillary electrophoresis using (CE) semipermanently coated fused silica capillaries has been applied for the analysis of recombinant somatropin [2]. The coating is generated by sequential adsorption of polybrene as a polycation and chondroitin sulfate A as a polyanion on the capillary inner walls. The coating is easily generated by using the rinse function of CE instrumentation.

Nguyet Thuy Tran and Myriam Taverna (eds.), *Capillary Electrophoresis of Proteins and Peptides: Methods and Protocols*, Methods in Molecular Biology, vol. 1466, DOI 10.1007/978-1-4939-4014-1_8,

In order to enhance the sample throughput of the separation system double-injection CE, where two somatropin samples are analyzed within a single run, has been applied [2].

1.1 Capillary Coating

CE has demonstrated its ability to provide excellent separation of proteins. Traditional slab gel electrophoresis techniques such as isoelectric focusing (IEF) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) tend to be replaced by CE. However, despite all the advantages that capillary electrophoresis offers, CE separation of proteins is often complicated since proteins tend to interact with the inner wall of silica capillary through electrostatic attraction, hydrogen bond, hydrophobic interactions, and van der Waals forces [3]. Protein adsorption process can be reversible, semi-reversible, or irreversible, depending on the structure and property of protein. It has been shown that even a slight protein-wall interaction leads to a significant reduction in the separation efficiency [4, 5].

The silanol groups on the surface of uncoated fused silica capillaries have pk_a values in the range of 2–5.3, being negatively charged at pH values higher than 3 [6]. Adsorption of proteins and peptides onto the capillary surface changes the zeta potential and, consequently, changes electroosmosis (EOF) [5]. Thus a controlled and constant electroosmotic flow improves the reproducibility of migration times. Several approaches have been developed to control the effects of capillary surface charges on the protein separation. These approaches include fine-tuning of electrophoresis conditions and modification of the capillary surface [7]. The ionization of the silanol groups can be suppressed using low-pH background electrolytes. However, extreme pH conditions may affect solubility and stability of proteins. The ionic interactions can also be controlled by adjusting the pH of the running buffer, since the net charge of a protein is positive at pH values lower than its isoelectric point (pI) and negative when the pH is above the pI. The chemical modification of the capillary wall is the most effective approach to eliminate protein adsorption and to manipulate electroosmosis [5, 7, 8]. Coating can influence the magnitude and direction of the EOF (μ_{eo}) and thereby apparent protein mobility, providing the possibility of selecting the best conditions for the protein separation. Chemical modification of the capillary walls can be achieved through dynamic or static coating methods (*see* Fig. 1). Tables 1 and 2 and Fig. 2 summarize some commonly used coating agents in CE [9–50]. The coating material should efficiently suppress adsorption of protein on the surface of capillary. It should also be pH stable, UV transparent, and applied over a wide range of electrophoretic conditions.

1.1.1 Dynamic Adsorbed Capillary Coating

In dynamic coating, the coating agents (*see* Table 1) are added into the BGE and thus the presence of the coating agent in the BGE keeps the coating on the capillary wall surface and changes its effective surface charge.

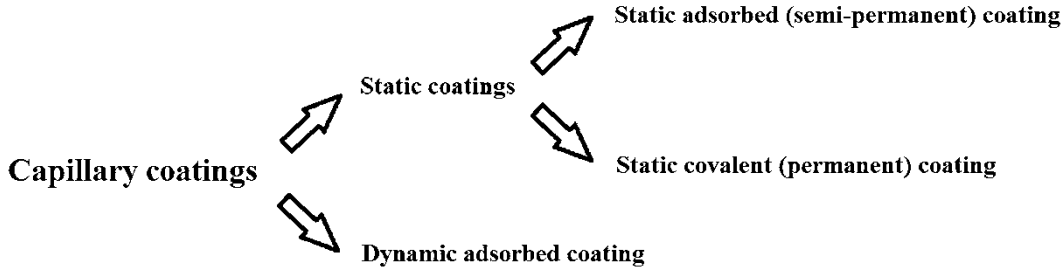

Fig. 1 The general coating strategies for the capillary coating in capillary electrophoresis. Dynamic coating agents are added into the running buffer. Static coating agents are attached to the surface through adsorption or by covalent bonding

Table 1
Some of the usual dynamic coating materials in CE

Coating material	Reference
Cetyltrimethylammonium bromide (CTAB)	[9]
β-D-Glycopyranoside	[10]
Triethanolamine	[11]
Polyethyleneimine (PEI)	[12]
Ethylamine	[13]
Triethylamine	[14]
Hydroxyethylcellulose (HEC)	[15]
Hydroxypropylmethylcellulose (HMPC)	[16]
Poly(vinyl alcohol) (PVA)	[17]
Poly(vinyl alcohol) dextrans	[18]
Polyethylene ether (Brij-35)	[19]
Poly(alkylene glycol) polymers	[20]
Poly(ethyleneglycol) (PEG)	[21]
Poly(ethylene oxide) (PEO)	[22]
Alkylamines and surfactants	[23]
1-Alkyl-3-methylimidazolium	[24]
Chitosan	[25]

Dynamic coated capillaries do not suffer from stability and durability issues, since the coating on the surface is permanently refreshed. However, the presence of charged coating agents in the BGE contributes to the conductivity of the BGE which generates joule heating. Furthermore, the presence of additives in the BGE may affect stability and solubility of proteins.

Table 2
Some of the usual permanent (static/covalent) and semipermanent (static/adsorption) coating materials in CE

Coating material	Coating type	Reference
Poly (acrylamide) and related derivatives	Permanent	[7, 26]
PEG coatings	Permanent	[27]
Poly(vinylpyrrolidone) (PVP)	Permanent	[28–30]
Cellulose derivatives	Permanent	[31]
Dextran	Permanent	[32]
Poly(vinylamine)	Permanent	[33]
Poly(vinylalcohol) (PVA)	Permanent	[34]
Tetradecyltrimethylammonium bromide	Semipermanent	[35]
Cetyltriethylammonium bromide	Semipermanent	[36]
Hydroxymethyl propylcellulose (HMPC)	Permanent	[37]
Hydroxy propylcellulose	Permanent	[38]
3-(Aminopropyl) trimethoxysilane (APS)	Permanent	[39]
Polybrene/dextran sulfate	Semipermanent	[40]
Polybrene, PEI	Semipermanent	[41, 42]
Polyethylene oxide (PEO)	Semipermanent	[22]
Polybrene/dextran sulfate	Semipermanent	[43]
Cellulose acetate	Semipermanent	[44]
Polybrene/chondroitin sulfate A	Semipermanent	[2]
Polybrene-dextran sulfate-Polybrene (PB-DS-PB)	Semipermanent	[45]
Poly(diallyldimethylammonium)/poly (styrene sulfonate)	Semipermanent	[46]
Polybrene/poly(vinylsulfonate) (PVS)	Semipermanent	[4, 35, 36, 45]
[(Acryloylamino)propyl]trimethylammonium chloride (BCQ)	Permanent	[47]
Epoxy-based coating	Permanent	[48]
Didecyldimethylammonium bromide (DDAB)	Semipermanent	[49]
Fibrinogen	Semipermanent	[50]

1.1.2 Static Coating of Fused Silica Capillaries

In order to overcome the effect of the coating material on the CE analysis static coating procedures have been developed. Static coating agents are not present in the running buffer. These coatings are permanently or semipermanently attached to the capillary surface by either adsorption (static-adsorption coating) or covalent bonds (static-covalent coating) (*see* Fig. 1 and Table 2).

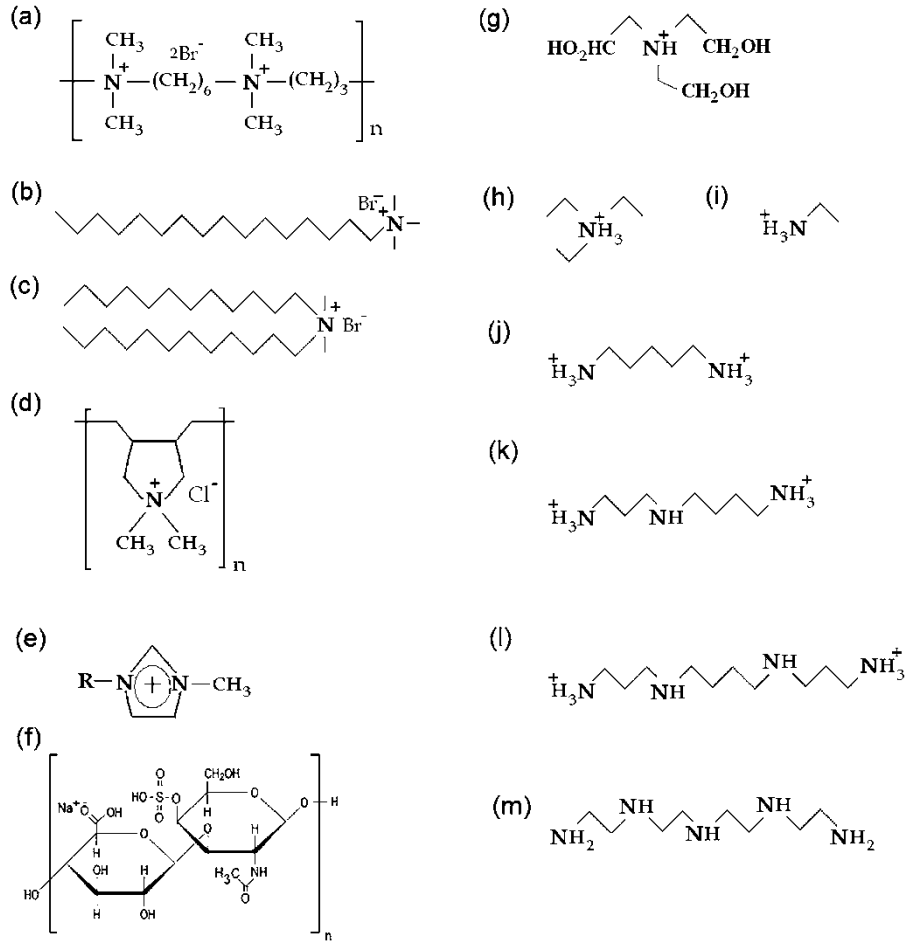

Fig. 2 Polymers and amines utilized for dynamic and static coating. (**a**) Hexamethonium bromide (Polybrene), (**b**) cetyltrimethylammonium bromide (CTAB), (**c**) didodecylmethylammonium bromide (DDAB), (**d**) poly(diallyldimethyl-ammonium chloride), (**e**) 1-alkyl-3-methylimidazolium cation, (**f**) chondroitin sulfate A, (**g**) triethanolamine, (**h**) triethylamine, (**i**) ethylamine, (**j**) cadaverine, (**k**) spermidine, (**l**) spermine, and (**m**) tetraethylenepentamine

Static Physically Adsorbed Coatings

Semipermanent or static adsorption coating is performed by simply flushing the capillary with a solution of an adsorptive coating agent [51]. The coating polymer is sufficiently adsorbed on to the surface through non-covalent interactions, e.g. electrostatic interactions. This creates a non-equilibrium condition which results in a successive desorption of the coating that must be thus frequently regenerated. The adsorbed polymers introduce neutral, positive, or even negative charges on the surface. This eliminates or changes the direction and/or magnitude of the EOF, as well as protein-surface interactions. Interaction between a hydrophilic neutral polymer and the surface is governed primarily by hydrogen bonds formed between functional groups of the polymer and protonated silanol groups.

The main advantages of semipermanent coating include the following: (1) The simplicity of the coating procedure. (2) The coating can easily be cleaned and regenerated by washing the capillary with sodium hydroxide or strong acids.

However, these coatings suffer from poor reproducibility because of successive desorption of the coating agent from the surface [42]. A small amount of coating agent may be added into the BGE in order to maintain the coating. Static adsorbed coatings could also be problematic for online MS analysis of proteins. In order to enhance the stability of static adsorbed coating a new approach based on multiple-layer coating was introduced [43]. In this approach the capillary is first rinsed with an initiator solution containing a polymeric polycation that is adsorbed on to the wall surface through ionic interactions. This step is followed by introduction of a solution containing polyanionic polymers that is forming the second and the negatively charged layer.

Static Covalent Capillary Coatings

In this approach the silanol groups present on the surface are covalently modified by coating material, e.g. polyacrylamide [7], cellulose derivatives [31], and dextran [32] (*see* Table 2). Covalently coated capillary cannot be regenerated and often has a limited lifetime. The advantage of this type of coating is that hydrophilic agent, which may be difficult to keep on the surface by dynamic or semi-static coatings, can be attached to the surface.

Permanent covalent coatings suffer, however, from several drawbacks, e.g., time-consuming and expensive coating procedures, the limited stability of the coating within a specific pH range, and the impossibility of recoating. The coating and thereby the capillary lifetime can be short and reproducibility of the coating from capillary to capillary may be poor which affect reproducibility of migration times.

1.2 Double-Injection Capillary Electrophoresis

Double-injection capillary zone electrophoresis (DICZE) is a mode of CE, which provides the possibility of analyzing two sequentially injected samples within a single run. The advantage of DICZE over the single-injection mode may be a reduction in the run-to-run variations in the migration times and thus in the peak areas, mainly caused by small changes in the EOF [52, 53]. In DICZE the inter-plug distance is regulated by applying an electrical field over the capillary for a predetermined period of time (t_{PE}) after the first injection. After the second injection, the separation is completed by electrophoresis for a time period corresponding to that in the single-injection mode [53].

2 Materials

All solutions should be prepared by using ultrapure water and reagents of analytical grade.

2.1 Sample Preparation

1. Somatropin-certified reference standard (CRS) should be purchased from the European Directorate for the Quality of Medicines & Health Care (EDQM, Strasburg, France).
2. The protein solution should be prepared freshly (*see* **Note 1**).
3. Prepare the sample solution by dissolving somatropin in water to a final concentration of 1.0 mg/mL (*see* **Note 2**).

2.2 CZE Running Protocol

1. Running buffer: 100 mM Ammonium phosphate at pH 6.0. Weigh 13.2 g ammonium phosphate dibasic and transfer to 1 L glass beaker. Add water to a volume of approximately 900 mL. Mix and adjust the pH with *O*-phosphoric acid 85 %. Transfer the buffer to a 1 L volumetric flask and make up to 1 L with water.
2. A fused silica capillary of dimensions (60 cm effective length and 70 cm total length) × 50 μm id (od 375 μm) is used (*see* **Note 3**).
3. Filter all solutions through 0.22 μm filters before use.

3 Methods

3.1 Capillary Conditioning and Coating

1. A new capillary is preconditioned with water (5 min), 0.1 M HCl (5 min), and 0.1 M NaOH (10 min), followed by approximately 5 mg/mL polybrene (PB) in water (5 min), 5 mg/mL chondroitin sulfate (CS) in water (5 min), and finally BGE for 10 min at 60 psi (413 kPa) (*see* Table 3).
2. Adjust the temperature of the capillary and sample storage at 30 and 10 °C, respectively. Detect the separated protein zones by UV detection at 200 nm.
3. Refresh running buffer after each ten separation cycles by either replacing the running buffer vials or switching to another buffer vials.
4. Regenerate the coating (at least every five runs) by washing the capillary by 0.1 M NaOH followed by PB and CS solutions as described above.

3.2 Double-Injection Capillary Electrophoresis

The p*I* of somatropin and its related proteins are in the range of 4.9–5.2 [54]. Somatropin and its related proteins are negatively charged under the separation conditions, i.e., at pH 6.0, being the same as the inner surface of fused silica capillaries. In contrast to the inner walls of the fused silica capillaries, the hydrophilic nature of the chondroitin sulfate A coating layer keeps somatropin and the related proteins away from the capillary surface, thereby improving the separation efficiency and precision of the determinations [2]. At the applied pH condition the negatively charged proteins will be pushed toward the cathodic end of the capillary by the strong EOF. Two sample injections are performed where the first

Table 3
Running method for the analysis of somatropin by capillary-zone electrophoresis in double-injection mod in PB/CS double-coated capillaries

Procedure	Inlet vial	Pressure (kPa)	Voltage (kV)	Time (s)
1. Wash	18 M Ω DW	413	0	300
2. Clean	0.1 M HCl	413	0	300
3. Clean	0.1 M NaOH	413	0	300
4. Coating (initiator)	Polybrene	413	0	300
5. Coating	Chondroitin sulfate A	413	0	300
6. Fill	Running buffer	413	0	600
7. Injection (I)	Sample load	5.5	0	10
8. Partial separation	Running buffer	0	10 or 17.4	900 (t_{PE})
9. Injection (II)	Sample	5.5	0	10
10. Separation	Running buffer	0	10 or 17.4	2700 1800
11. Peak integration and data analysis				

Steps 1 through 5 are applied for conditioning and coating of a new capillary. Between-run conditioning of the capillary is carried out by flushing the capillary with the BGE (step 6)

applied plug is electrophoresed for 15 min prior to the second injection (*see* Fig. 3). The analyses are performed according to the following procedure:

1. Wash the coated capillary with the running buffer for 5 min (413 kPa) prior to the sample injection.
2. Inject the somatropin sample (1 mg/mL). Injections are performed hydrodynamically by applying a pressure of 0.8 psi (5.5 kPa) at the cathodic end of the capillary for 10 s (*see* **Notes 1** and **5**).
3. Apply a potential of between 10 and 17.4 kV across the capillary (*see* **Note 6** and ref. [2]).
4. Stop the separation after 15 min to prepare for the second injection.
5. Perform the second injection (from the same injection vial as in **step 2** or another sample vial with the same content) (*see* **Notes 5** and **7**).
6. Apply the same voltage as in step 3 across the capillary for 45 min. The peaks will be recorded by the detector (*see* ref. [2] and Fig. 3). The peaks originating from the compounds in the first injection will elute within the first part of the electropherogram (*see* Fig. 3).
7. Wash the capillary with the BGE between two runs.

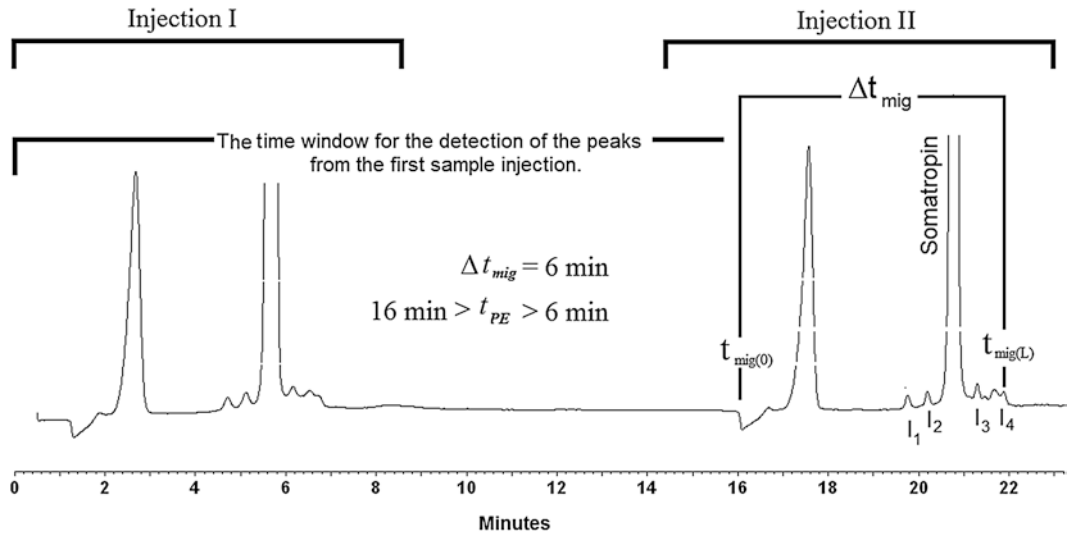

Fig. 3 Analysis of somatropin reference standard in single- (**a**) and double-CZE modes (**b**). Running conditions: a PB/CS double-coated capillary of dimensions 70 × 60 cm × 50 μm was used. Separation was performed at 17.4 kV (90 μA) at 30 °C. Prior to the second injection, the first injected sample was electrophoresed at 17.4 kV for 15 min (t_{PE})

3.3 Purity Determination

1. Integrate the peaks eluting prior to the somatropin peak, i.e., I_1 and I_2, and the peaks eluting after the main peak, i.e., I_3 and I_4 (*see* Fig. 3 and **Note 6**). The purity of somatropin is expressed as its peak area present relative to the total peak areas of all the proteins, including somatropin. Impurity limits according to European Pharmacopoeia [55]: deamidated forms: maximum 5.0%; any other impurity: for each impurity 2.0%; total: maximum 10.0%.
2. Peak I_4 corresponds to the deamidated forms, eluting as a doublet. These partially resolved peaks are integrated as a single peak.

3.4 Result Treatment

1. Analyze the somatropin sample in single-injection mode as described above (*see* Table 3).
2. The analysis time period will approximately be 30 or 45 min when the applied voltage is 10 or 17.4 kV, respectively.
3. Calculate the difference between the negative peak of water ($t_{\mathrm{mig}(0)}$) and the last eluting peak ($t_{\mathrm{mig}(I4)}$), i.e., I_4 (*see* Fig. 3):

$$\Delta t_{\mathrm{mig}} = t_{\mathrm{mig}(0)} - t_{\mathrm{mig}(I4)} \quad (1)$$

4. The time period for the partial electrophoresis (t_{PE}) should be longer than Δt_{mig} and shorter than $t_{\mathrm{mig}(0)}$, since the peaks from the first injection are eluting within a time window corresponding to the first eluting peak from the second injection, i.e., $t_{\mathrm{mig}(0)}$ (*see* **Notes 4**, **6**, and **8**):

$$t_{\mathrm{mig}(0)} > t_{\mathrm{PE}} > \Delta t_{\mathrm{mig}} \quad (2)$$

5. Analyze the result by calculating the peak area (A) ratios by means of the following formula:

$$A_i\% = 100 \times A_i / £A \tag{3}$$

where

$$£A = A_{(I1)} + A_{(I2)} + A_{(\text{somatropin})} + A_{(I3)} + A_{(I4)} \tag{4}$$

4 Notes

1. Exposure of the somatropin solution to heat and light results in degradation, e.g., oxidation and deamidation.
2. Somatropin-certified reference standard (CRS) contains salts in addition to somatropin; therefore all the content of the CRS vial should be dissolved in a fixed volume of water to bring about a concentration of 1 mg/mL. It is difficult to get an accurate weight of somatropin by weighting a quantity.
3. In order to avoid overloading and improve the reproducibility of the injection volume, the ends of the capillary, especially the cathodic end, should be cut carefully. A flat cut can be accomplished when the capillary ends are cut using an SGT Shortix capillary column cutter (Middelburg, the Netherlands). The ends of the capillary may be burned in order to clean the capillary ends from polyimide coating.
4. A t_{PE} shorter than Δt_{mig} results in inter-plug interference. When the t_{PE} is longer than $t_{mig(0)}$ all or some of the peaks, depending on the length of the t_{PE}, from the first injection will not show up on the electropherogram.
5. Make sure that there is no air bubble at the bottom of the injection vial, i.e., the PCR vial. Injection time and pressure can be manipulated to get an acceptable peak height and efficiency.
6. Higher voltage, e.g., higher than 17.4 kV, can generate more heat which can impair peak shape and separation efficiency. Application of 17.4, which generates a current of 90 μA, reduces the analysis time from 45 min (at 10 kV) to 30 min.
7. The method is developed to analyze somatropin reference standard; however, it may be applied to the analysis of any somatropin formulation if no interfering compounds were present in the formulation (*see* ref. [52]).
8. Integration can be carried out automatically; however, it is important to check that it has been performed properly. The calculations are performed manually in Excel.

References

1. Ameeta M, Hindmarsh PC (2002) The use of somatropin (recombinant growth hormone) in children of short stature Pediatric Drugs 4:37–47
2. Amini A (2013) Separation of somatropin charge variants by multiple-injection CZE with polybrene/chondroitin sulfate A double-coated capillaries. J Sep Sci 36:2686–5690
3. Doherty EAS, Meagher RJ, Albarghouthi MN, Barron AE (2003) Microchannel wall coatings for protein separations by capillary and chip electrophoresis. Electrophoresis 24:34–54
4. Green JS, Jorgenson JW (1989) Minimizing asorption of proteins on fused silica in capillary zone electrophoresis by the addition of alkali metal salts to the buffers. J Chromatogr A 478:63–70
5. Towns JK, Regnier FE (1992) Impact of polycation adsorption on efficiency and electroosmotically driven transport in capillary electrophoresis. Anal Chem 64:2473–2478
6. Schwer C, Kenndler E (1991) Ectrophoresis in fused-silica capillaries: the influence of organic solvents on the electroosmotic velocity and the zeta potential. Anal Chem 63:1801–1807
7. Hjertén S (1985) High-performance electrophoresis: elimination of electroendosmosis and solute adsorption). J Chromatogr 347:191–198
8. Lauer HH, McManigill D (1986) Capillary zone electrophoresis of proteins in untreated fused silica tubing. Anal Chem 58:166–170
9. Chiari M, Damin F, Melis A, Conssoni R (1998) Separation of oligonucleotides and DNA fragments by capillary electrophoresis in dynamically and permanently coated capillaries, using a copolymer of acrylamide and β-D-glucopyranoside as a new low viscosity matrix with high sieving capacity. Electrophoresis 19:3154–3159
10. Wang Z, Chen Y, Yuan H et al (2000) (Preparation and characterization of calixarene-coated capillaries for capillary electrophoresis. Electrophoresis 21:1620–1624
11. Dobashi A, Hamada M (1999) Chiral separation of dansyl-DL-amino acids with micellar systems containing copper (II) ion and N-n-dodecyl-L-proline in electrokinetic capillary chromatography. Electrophoresis 20:2761–2765
12. Erim FB, Cifuentes A, Poppe H, Kraak JC (1995) Performance of a physically adsorbed high-molecular-mass polyethyleneimine layer as coating for the separation of basic proteins and peptides by capillary electrophoresis. J Chromatogr A 708:356–361
13. Legaz ME, Pedrosa MM (1996) Effect of polyamines on the separation of ovalbumin glycoforms by capillary electrophoresis. J Chromatogr A 719:159–170
14. Corradini P, Rhomberg A, Corradini C (1994) Electrophoresis of proteins in uncoated capillaries with amines and amino sugars as electrolyte additives. J Chromatogr A 661:305–313
15. Olivieri E, Sebastiano R, Citterio A et al (2000) Quantitation of protein binding to the capillary wall in acidic, isoelectric buffers and means for minimizing the phenomenon. J Chromatogr A 894:273–280
16. Linder H, Helliger W, Sarg B, Meraner C (1995) (Effect of buffer composition on the migration order and separation of histone H1 subtypes. Electrophoresis 16:604–610
17. Kleemiss MH, Gilges M, Schomburg G (1993) Capillary electrophoresis of DNA restriction fragments with solutions of entangled polymers. Electrophoresis 14:515–522
18. Towns JK, Regnier FE (1991) Capillary electrophoretic separations of proteins using non-ionic surfactant coatings. Anal Chem 63:1126–1132
19. Salmanowicz BP (1995) Capillary electrophoresis of seed albumins from vicia species using uncoated and surface-modified fused silica capillaries. Chromatographia 41:99–106
20. Oneill K, Shao XW, Zhao ZX et al (1994) Capillary electrophoresis of nucleotides on ucon-coated fused silica columns. Anal Biochem 222:185–189
21. Chen SH, Tzeng RT (1999) Polymer solution-filled column for the analysis of antisense phosphorothioates by capillary electrophoresis. Electrophoresis 20:547–554
22. Iki N, Yeung ES (1996) Non-bonded poly(ethylene oxide) polymer-coated column for protein separation by capillary electrophoresis. J Chromatogr A 731:273–282
23. Tsuda T (1987) Modification of electroosmotic flow with cetyltrimethylammonium bromide in capillary zone electrophoresis. J Sep Sci 10:622–624
24. Jiang T-F, Gu Y-L, Liang B et al (2003) Dynamically coating the capillary with 1-alkyl-3-methylimidazolium-based ionic liquids for separation of basic proteins by capillary electrophoresis. Anal Chim Acta 479:249–254
25. Yao YJ, Li SFY (1991) Capillary zone electrophoresis of basic proteins with chitosan as a capillary modifier. J Chromatogr A 663: 97–104

26. Cobb KA, Dolnik V, Novotny M (1990) Electrophoretic separations of proteins in capillaries with hydrolytically-stable surface structures. Anal Chem 62:2478–2483
27. Nashabeh W, El Rassi Z (1991) Capillary zone electrophoresis of proteins with hydrophilic fused-silica capillaries. J Chromatogr A 559:367–383
28. Smith JT, El Rassi Z (1993) Capillary zone electrophoresis of biological substances with fused silica capillaries having zero or constant electroosmotic flow. Electrophoresis 14:396–406
29. McCormick RM (1998) Capillary zone electrophoretic separation of peptides and proteins using low pH buffers in modified silica capillaries. Anal Chem 60:2322–2328
30. Yao XW, Wu D, Regnier FE (1993) Manipulation of electroosmotic flow in capillary electophoresis. J Chromatogr A 636:21–29
31. Shen Y, Smith RD (2000) High-resolution capillary isoelectric focusing of proteins using highly hydrophilic-substituted cellulose-coated capillaries. J Microcol 12:135–141
32. Hjertén S, Kubo K (1993) A new type of pH- and detergent-stable coating for elimination of electroendosmosis and adsorption in (capillary) electrophoresis. Electrophoresis 14:390–395
33. Chiari M, Coriotti L, Crini G, Morcellet M (1999) Poly(vinylamine)-coated capillaries with reversed electroosmotic flow for the separation of organic anions. J Chromatogr A 836:81–91
34. Belder D, Deege A, Husmann H et al (2001) Cross-linked poly(vinyl alcohol) as permanent hydrophilic column coating for capillary electrophoresis. Electrophoresis 2001(22):3813–3818
35. Bendahl L, Hansen SH, Gammelgaard B (2001) Capillaries modified by noncovalent anionic polymer adsorption for capillary zone electrophoresis, micellar electrokinetic capillary chromatography and capillary electrophoresis mass spectrometry. Electrophoresis 22:2565–2573
36. Graul TW, Schlenoff JB (1999) Capillaries modified by polyelectrolyte multilayers for electrophoretic separations. Anal Chem 71:4007–4013
37. Aguilar C, Hofte AJP, Tjaden UR, van der Greef J (2001) Analysis of histones by on-line capillary zone electrophoresis–electrospray ionisation mass spectrometry. J Chromatogr A 926:57–67
38. Busnel JM, Desoroix S, Le Saux T et al (2006) Protein tryptic digests analyzed by carrier ampholyte-based capillary electrophoresis coupled to ESI-MS. Electrophoresis 27:1481–1488
39. Wahl JH, Gale DC, Smith RD (1994) Sheathless capillary electrophoresis-electrospray ionization mass spectrometry using 10 μm I.D. capillaries: analyses of tryptic digests of cytochrome c. J Chromtogr A 659:217–222
40. Katayama H, Ishihama Y, Asakawa N (1998) Stable cationic capillary coating with successive multiple ionic polymer layers for capillary electrophoresis. Anal Chem 70:5272–5277
41. Amini A, Olofsson IM (2004) Analysis of calcitonin and its analogues by capillary zone electrophoresis and matrix-assisted laserdesorption ionization time-of-flight mass spectrometry. J Sep Sci 27:675–685
42. Cordova E, Gao J, Whitesides GM (1997) Noncovalent polycationic coatings for capillaries in capillary electrophoresis of proteins. Anal Chem 69:1370–1379
43. Katayama H, Ishihama Y, Asakawa N (1998) Stable capillary coating with successive multiple ionic polymer layers. Anal Chem 70:5254–5260
44. Busch MHA, Kraak JC, Poppe H (1995) Cellulose acetate-coated fused-silica capillaries for the separation of proteins by capillary zone electrophoresis. J Chromatogr A 695:287–296
45. Haselberg R, Brinks V, Hawe A, de Jong GJ (2011) Capillary electrophoresis-mass spectrometry using non-covalently coated capillaries for the analysis of biopharmaceuticals. Anal Bioanal Chem 400:295–303
46. Catai JR, Toraño JS, Jongen PMJM et al (2007) Analysis of recombinant human growth hormone by capillary electrophoresis with bilayer-coated capillaries using UV and MS detection. J Chromatogr B 852:160–166
47. Bateman KP, White RL, Thibault P (1997) Disposable emitters for on-line capillary zone electrophoresis/nanoelectrospray mass spectrometry. Rapid Commun Mass Spectrom 11:307–315
48. Bao JJ (2000) Separation of proteins by capillary electrophoresis using epoxy based hydrophilic coating. J Liq Chro Rel Technol 23:61–78
49. Melanson EM, Baryla NE, Lucy CA (2000) Double-chained surfactants for semipermanent wall coatings in capillary electrophoresis. Anal Chem 72:4110–4114
50. Van Tassel PR, Miras D, Hagege A et al (1996) Control of protein adsorption in capillary electrophoresis via an irreversibly bound protein coating. J Colloid Interface Sci 183:269–273
51. Lucy CA, MacDonal AM, Gulcev MD (2008) Non-covalent capillary coatings for protein separations in capillary electrophoresis. J Chromatogr A 1184:81–105

52. Amini A (2014) Double injection capillary electrophoresis for the identification of analytes. Electrophoresis 35:2915–2921
53. Amini A, Lodén H, Pettersson C, Arvidsson T (2008) Principles for different modes of multipleinjection CZE. Electrophoresis 29: 3952–3958
54. Dupin P, Galinou F, Bayol A (1995) Analysis of recombinant human growth hormone and its related impurities by capillary electrophoresis. J Chromatogr A 707:396–400
55. Somatropin for injection. European Pharmacopoeia, 8th Edition 2016 (8.8) (01/2008:0952)

Chapter 9

Poly(*N,N*-Dimethylacrylamide)-Based Coatings to Modulate Electroosmotic Flow and Capillary Surface Properties for Protein Analysis

Laura Sola, Marina Cretich, and Marcella Chiari

Abstract

Capillary electrophoresis (CE) is one of the most powerful techniques for the separation of biomolecules. However, the separation efficiency of proteins in CE is often compromised by their tendency to interact with the silanol groups on the surface of the inner capillary and by an uncontrolled electroosmotic flow. Herein, we report on the use of novel hydrophilic polymeric coatings that can modulate the properties of the capillary walls. The novelty of these poly(*N,N*-dimethylacrylamide)-based copolymers relies on the simultaneous presence of chemically reactive groups (*N*-acryloyloxysuccinimide and glycidyl methacrylate) and silane groups in the backbone, which results in highly stable films due to the covalent reaction between the polymer and the glass silanols. A careful optimization of monomer concentration confers anti-fouling properties to the polymer coatings, and thus allows for highly efficient acidic and alkaline protein separations. Furthermore, the presence of these monomers makes it possible to modulate the electroosmotic flow from negligible to reduced values, depending on the desired application.

Key words Capillary electrophoresis, Polymeric coatings, Physisorbed, Chemisorbed, Electroosmotic flow, Protein separation

1 Introduction

Capillary electrophoresis (CE) is a powerful analytical technique that combines the advantages of electrophoretic separation with automation, allowing for a fast and efficient separation of a wide variety of compounds, including chemical, pharmaceutical, and biological samples. However, the CE separation of proteins can be complicated because of their tendency to adsorb onto the negatively charged glass walls due to Coulombic interactions [1, 2]. Furthermore, uncontrolled electroosmotic flow (EOF) drastically affects the mobility of the analyte, leading either to loss of resolution due to short migration times or loss of efficiency due to excessively long migration times. For most CE applications, the control or suppression of EOF is necessary to fully exploit the applicability

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of the technique. In some cases, for instance at high pH, the EOF may be too high, resulting in the elution of analytes before the separation has taken place. Numerous approaches have been evaluated to minimize these problems, such as using buffers with extreme pH values [3, 4], high ionic strengths [5], or zwitterionic additives. In other applications, such as CE coupled to mass spectrometry (MS), a stable coating is crucial for achieving reproducibility and low background noise [6–8]. As a consequence, an effective modification of the surface is necessary to obtain highly efficient and reproducible separations. Among the several techniques that have been developed to control and modulate the properties of the capillary wall, high-quality polymer coatings have shown a crucial role [9].

Permanent polymeric coatings can be achieved by introducing surface anchoring groups through organosilanization, followed by the covalent attachment of polymers [10]. However, the stability of the attachment via this approach relies on the quality of the silanization process, which is often difficult to control. Alternative approaches can produce polymeric coatings in a more robust and less cumbersome manner. When silica surfaces are exposed to very dilute solutions of certain polymers, they develop a dense polymer layer that involves hydrogen bonding, as well as monopolar, dipolar, or hydrophobic forces. Unfortunately, unless a strategy is adopted to stabilize the polymer attachment, polymer chains may be slowly released from the surface, resulting in loss of performance over time. Our group has always been involved into this research area, particularly in the field of adsorbed polymeric coatings. In fact, our laboratory has introduced polymer coatings obtained by the adsorption of dimethylacrylamide and allylglycidyl ether copolymers (EPDMA) [11]. Poly(DMA) self-adsorbs onto the capillary walls and does not seem to be removed from the surface by an aqueous buffer because of the solubility of the polymer in water, and because of the formation of a large number of hydrogen bonds with the silica surface [11]. The addition of epoxy groups to the polymer backbone (Fig. 1a) showed a significant improvement of the polymer adsorption and stability, leading to the possibility of using this coating also in absence of a replacing polymer into the buffer. Moreover, the molecular weight of

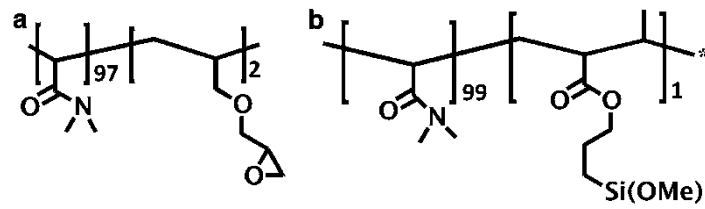

Fig. 1 Chemical structures of the DMA-based copolymers: (**a**) EPDMA; (**b**) poly(DMA-MAPS)

EPDMA was found to strongly impact the stability of the layer because the sticking energy per chain increases in proportion to the number of monomer units.

A further improvement on the coating stabilization has been introduced by adding a silane moiety (3-(trimethoxysilyl) propyl methacrylate, MAPS) to the polymer backbone: the copolymer, named poly(DMA-MAPS), is physisorbed on the wall and then grafted by thermal curing (Fig. 1b) [12]. An evolution of this polymer was employed in microarray technologies [13, 14] to covalently bind proteins and DNA to flat surfaces. This polymer contains the following functionalities: (1) a *N,N*-dimethylacrylamide (DMA) backbone that binds to the surface by weak, non-covalent interactions such as hydrogen bonding, Van der Waals or hydrophobic forces, (2) a pending silane hydrolyzable monomer (MAPS), which promotes the condensation of the polymer with surface silanols, and (3) a chemically reactive monomer, *N*-acryloyloxysuccinimide (NAS). This copolymer, poly(DMA-NAS-MAPS), forms a coating with characteristics of irreversibility on a variety of materials [15–17] through a process that conjugates physi- and chemisorption [13].

In this work, we utilize poly(DMA-NAS-MAPS) and a similar polymer, poly(DMA-GMA-MAPS), wherein the NAS functional groups have been replaced with glycidyl methacrylate (GMA), to produce stable hydrophilic coatings that suppress EOF and improve protein separation in CE [18, 19]. In Fig. 2, the chemical structures of these DMA-based copolymers are shown.

The presence of functional monomers such as NAS (Fig. 2a) or GMA (Fig. 2b) makes possible to modulate the EOF from low to negligible values. In fact, the functional group NAS can be

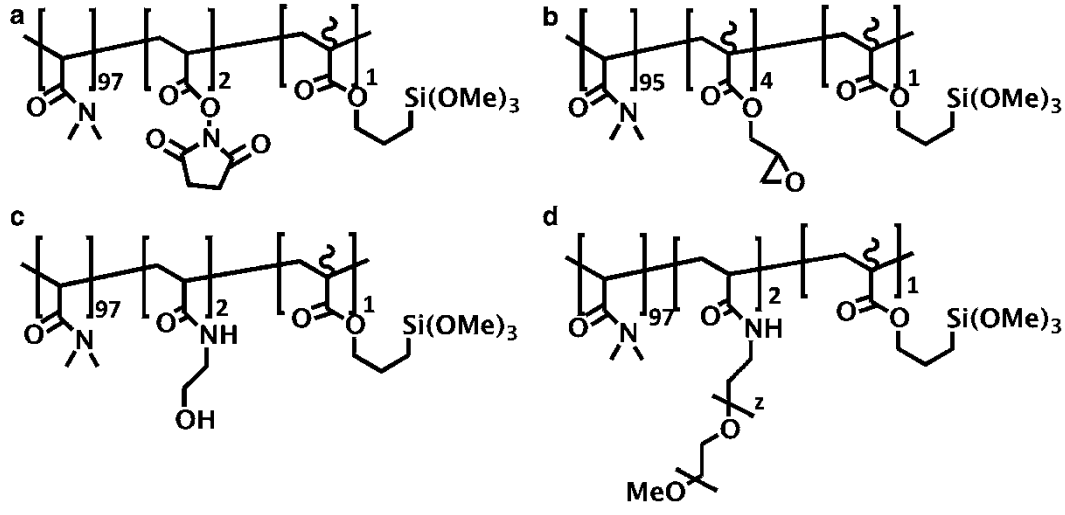

Fig. 2 Chemical structure of (**a**) poly(DMA-NAS-MAPS), (**b**) poly(DMA-GMA-MAPS), (**c**) poly(DMA-NAS-MAPS) after the blocking step with ethanolamine, and (**d**) poly(DMA-NAS-MAPS) after the blocking step with amino-PEG

exploited to further modify the polymer layer to increase the shielding of the negative charges on the glass wall: a simple surface reaction with molecules containing amino functionalities (such as ethanolamine or amino-PEG) converts NAS into unreactive moieties, which improves the antifouling properties of the surface without interfering with the stability of the attachment. The rationale behind using ethanolamine and amino-PEG as blocking agents is related to the ease of the nucleophilic substitution reaction, which is conducted quickly under diluted aqueous conditions. Figure 2 shows the chemical structure of poly(DMA-NAS-MAPS) after the blocking step with ethanolamine (Fig. 2c) and amino-PEG (Fig. 2d). To avoid the blocking step procedure, the functional monomer can be substituted with glycidyl methacrylate (GMA), generating a polymer, poly(DMA-GMA-MAPS), that produces a hydrophilic coating with similar characteristics to those of poly(DMA-NAS-MAPS). Although glycidyl methacrylate (GMA) contains oxirane groups, which are reactive toward amino groups in proteins [20, 21], no reaction with proteins was observed using this coating. This is likely due to the low GMA molar content in the polymer and the fact that the oxirane moieties are oriented toward surface silanols and contribute to increase the stability of the film through the formation of additional covalent bonds with the silanols.

To demonstrate the ability of these coatings to modulate the capillary surface properties, measurements of EOF at different pH values were performed using the pressure mobilization method [22] (Fig. 3).

Examples of alkaline and acidic proteins were separated to demonstrate the performance of these coatings. In both cases, efficient separations were observed, with low retention of proteins on the capillary walls. The stability of the coating was demonstrated by the profile of twenty consecutive runs, which shows no significant modifications in the peak shape or retention time.

2 Materials

2.1 Synthesis of Poly(DMA-NAS-MAPS) and Poly(DMA-GMA-MAPS)

1. *N,N*-Dimethylacrylamide (DMA) filtered on aluminum oxide: fill a chromatography column with aluminum oxide for 1/3 of its height, then pour 20 mL of DMA on top of the aluminum oxide and elute it by applying positive pressure, from the top of the column (*see* **Note 1**).
2. 3-(Trimethoxysilyl)propyl methacrylate (MAPS) (*see* **Note 2**).
3. *N*-Acryloyloxysuccinimide (NAS): synthesized as reported by Mammen et al. [23] (*see* **Note 3**).
4. α,α′-Azoisobutyronitrile (AIBN): the final concentration of the initiator into the reaction mixture is 2 mM (*see* **Note 4**).

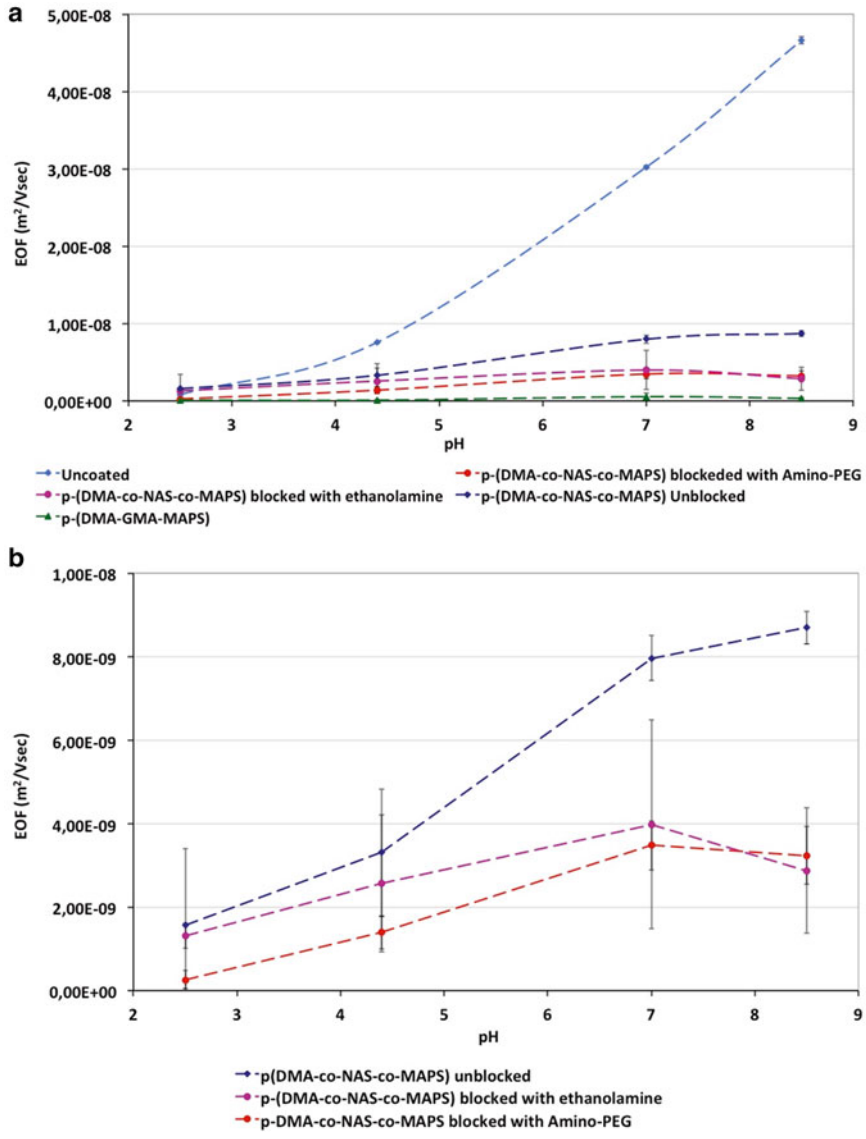

Fig. 3 EOF measurements performed using buffers at constant ionic strength (18 mM) at pH 2.5, 4.4, 7.0, and 8.5. EOF was measured in a 50 μm ID capillary, 54 cm total length, 44 cm to detection window using the pressure mobilization method [22]: (**a**) comparison of the EOF measured in coated and uncoated capillaries; (**b**) comparison of the EOF measured in capillaries coated with poly(DMA-NAS-MAPS) before and after blocking the functional monomer. Adapted from Sola L. et al. [19] with permission

2.2 Coating of Fused Silica Capillaries with Poly (DMA-NAS-MAPS) and Poly (DMA-GMA- MAPS)

1. Fused silica capillary. This method can be applied to any desired capillary length and diameter.
2. 1 M Sodium hydroxide solution: dissolve 20 g of sodium hydroxide in 500 mL of DI water.
3. 0.1 M Hydrochloric acid solution: add 4.1 mL of 12 N hydrochloric acid to 500 mL of DI water.

4. 1.6 M Ammonium sulfate (40% saturation level): add 121 g of ammonium sulfate to 500 mL of DI water.
5. Prepare the polymer coating solution using poly(DMA-NAS-MAPS) and poly(DMA-GMA-MAPS) synthesized as described in Subheadings 3.1 and 3.2. Dissolve poly(DMA-NAS-MAPS) or poly(DMA-GMA-MAPS) in DI water to a final concentration of 2% w/v and then dilute 1:1 with a 1.6 M water solution of ammonium sulfate (*see* **Note 5**). For example, to prepare 1 mL of coating solution, weight 10 mg of the copolymer and dissolve it in 500 μL of DI water. When it is completely solubilized, add 500 μL of the ammonium sulfate solution.

2.3 Blocking of Poly (DMA-NAS-MAPS) Functional Groups (See Note 6)

1. 50 mM Ethanolamine solution in 0.1 M TRIS/HCl, pH 9: dissolve 1.5 mL of ethanolamine in 448.5 mL of DI water and add 50 mL of a 1 M TRIS/HCl buffer, pH 9. To prepare the 1 M TRIS/HCl buffer, pH 9, dissolve 30.285 g of TRIZMA Base in 200 mL of DI water. Titrate with 5 M HCl to pH 9 and add DI water to the final volume of 250 mL.
2. Methoxy polyethylene glycol amine solution (amino-PEG, 2000, extent of labeling: ≥0.4 mmol/g NH_2 loading): dissolve 20 mg of amino-PEG in 150 mM H_3PO_4-NaOH buffer, pH 8.5. To prepare the phosphate buffer, add 2.18 mL of concentrate phosphoric acid to 200 mL of DI water. Titrate with a solution of 1 M NaOH to pH 8.5 and add DI water to the final volume of 250 mL.

2.4 Electroosmotic Flow Measurement

The dimensions of the fused silica capillaries are: 50 μm ID, 54 cm total length, and 44 cm to detection window.

1. Prepare running buffers (all at a constant ionic strength of 18 mM).
   - 25 mM H_3PO_4-14.4 mM NaOH pH 2.5: Dissolve 6.25 mL of a 1 M stock solution of H_3PO_4 in 200 mL of DI water. Add 3.62 mL of a 1 M stock solution of NaOH and add DI water to a final volume of 250 mL.
   - 44 mM 6-EACA-39.5 acetic acid pH 4.4: Dissolve 1.44 mg of 6-EACA in 200 mL of DI water. Add 0.57 mL of glacial acetic acid and add DI water to a final volume of 250 mL.
   - 10 mM H_3PO_4–14 mM NaOH pH 7.0: Dissolve 2.50 mL of a 1 M stock solution of H_3PO_4 in 200 mL of DI water. Add 3.50 mL of a 1 M stock solution of NaOH and add DI water to a final volume of 250 mL.
   - 31.5 mM bicine–52 mM TRIS pH 8.5 Dissolve 1.28 mg of bicine in 200 mL of DI water. Add 1.57 mg of Trizma base and add DI water to a final volume of 250 mL.
2. Solution of neutral marker: 0.8% w/v aqueous solution of acrylamide is dissolved in each buffer.

2.5 Alkaline Protein Separation

1. Alkaline protein mix: lysozyme, cytochrome C, and ribonuclease A. Dissolve each protein in DI water to a final concentration of 1 mg/mL.
2. Capillary (total length 110 cm, length to detection window 100 cm, ID 30 μm) coated with poly (DMA-NAS-MAPS) as reported in Subheading 2.2 and blocked with ethanolamine or amino-PEG as reported in Subheading 2.3.
3. Running buffer: 25 mM H_3PO_4-NaOH pH 3.1: Dissolve 6.25 mL of a 1 M stock solution of H_3PO_4 in 200 mL of DI water. Titrate with a 1 M stock solution of naH until the desired pH value. Add DI water to a final volume of 250 mL.

2.6 Acidic Protein Separation

1. Acidic protein mix: soy bean trypsin inhibitor, β-lactoglobuline, α-lactalbumine, carbonic anhydrase. Dissolve each protein in DI water to a final concentration of 1 mg/mL.
2. Capillary (total length 32 cm, length to detection window 22 cm, ID 30 μm) coated with poly (DMA-GMA-MAPS) as reported in Subheading 2.2.
3. Running buffer: 31.5 mM bicine–52 mM TRIS pH 8.5. Dissolve 1.28 mg of bicine in 200 mL of DI water. Add 1.57 mg of Trizma base and add DI water to a final volume of 250 mL.

3 Methods

3.1 Synthesis of Poly (DMA-NAS-MAPS) and Poly (DMA-GMA-MAPS)

The copolymer made of DMA (with 97% molar percentage), NAS (2% molar percentage), and 3-(trimethoxysilyl)propyl methacrylate (MAPS, 1% molar percentage) is synthesized by free radical copolymerization. The concentration of the monomer feed in the solvent is 20% w/v.

The composition of the poly (DMA-GMA-MAPS) is the same as the poly (DMA-NAS-MAPS) and it is synthesized with the same total monomer concentration.

1. In a 250 mL three neck round bottom flask, equipped with condenser, magnetic stirrer, and helium connection, degas 20.00 mL of anhydrous tetrahydrofuran (THF) by purging helium for 15 min (*see* **Note 7**).
2. Add *N,N*-dimethylacrylamide (DMA, 4.00 g, 4.00×10^{-2} mol; *see* **Note 1**), NAS (0.14 g, 8.30×10^{-4} mol; *see* **Note 3**) or GMA (0.23 g, 1.60 mmol) and the initiator α,α′-azoisobutyronitrile (AIBN, 1.30×10^{-2} g, 7.90×10^{-5} mol; *see* **Note 4**) into the flask, continuing purging helium (*see* **Note 8**).
3. Add 3-(trimethoxysilyl)propyl methacrylate (MAPS, 1.03×10^{-1} g, 4.15×10^{-4} mol; *see* **Note 2**) using a syringe.
4. Remove the gas inlet and heat the solution to 65 °C for 2 h, while stirring, under helium atmosphere (*see* **Note 9**).

5. After the polymerization is completed, dilute 1:1 by adding 20.00 mL of anhydrous THF (*see* **Note 10**) and stir for 5 min.
6. Precipitate the polymer by slowly dripping (*see* **Note 11**) the reaction mixture into a large excess of petroleum ether (about 1:10 by volume), while stirring.
7. Filter the obtained white solid on a Buchner funnel (*see* **Note 12**).
8. Dry the copolymer under vacuum for 1–2 h at room temperature.
9. Store it at −20 °C in a dry environment.

3.2 Coating of Fused Silica Capillaries with Poly (DMA-NAS-MAPS) and Poly (DMA-GMA- MAPS)

The protocol is composed by two parts: the coating follows an initial pretreatment procedure. To fill the capillaries use a positive nitrogen pressure (4.5 bar) by connecting the capillary to a nitrogen or compressed air source.

1. Flow a 1 M NaOH solution into the capillary for 30 min.
2. Rinse with DI water for 10 min (*see* **Note 13**).
3. Flow a 0.1 N HCl solution into the capillary for 1 h.
4. Rinse with DI water for 10 min (*see* **Note 13**).
5. Fill the capillary with the coating solution applying a positive nitrogen pressure for 10 min, then stop the pressure and allow the solutions to remain in the capillaries for additional 15 min.
6. Rinse the capillary with DI water for 10 min (*see* **Note 13**).
7. Empty completely the capillary using a positive nitrogen pressure.
8. Cure the coated capillary at 80 °C for 30 min (*see* **Note 14**).

3.3 Blocking of Poly (DMA-NAS-MAPS) Functional Groups

The robustness of the coatings is due not only to the presence of silanol groups, but also to the presence of functional groups (NAS and GMA) that enhance the solidity of the film by reacting with surface silanols. To increase the performance of poly(DMA-NAS-MAPS), its functional group (NAS) must be blocked before use, to avoid protein interaction with the surface. Considering the high reactivity of this monomer toward nucleophiles, a simple reaction with molecules containing amino groups (for example ethanolamine or amino-modified PEG) transforms NAS into unreactive moities, improving the antifouling properties of the coating. The second polymer, poly(DMA-co-GMA-co-MAPS), does not require blocking as the oxirane groups of GMA showed no reactivity toward protein during the separations. This is likely due to the low GMA molar content in the polymer and the fact that the oxirane moieties are oriented toward surface silanols.

The blocking procedure is similar for both blocking agents (ethanolamine and amino-modified PEG).

1. Flow the blocking solution (ethanolamine or amino-PEG) into the capillary using a positive nitrogen pressure (4.5 bar) for 10 min (*see* **Notes 6** and **13**).
2. Stop the pressure and incubate the filled capillary for 30 min at 50 °C; there is no need to seal to capillary extremities.
3. Rinse with DI water for 10 min (*see* **Note 13**).
4. Empty completely the capillary using a positive nitrogen pressure (*see* **Notes 14** and **15**).

3.4 Electroosmotic Flow Measurement

Electroosmotic flow was measured using the pressure mobilization method [22] for both coated and uncoated capillaries. Applied pressure, times, and voltage are those reported by Williams and Vigh [22].

These coatings modulate the capillary surface properties, as demonstrated by measurements of EOF at different pH values. Figure 3 demonstrates that both polymers result in a very efficient and permanent coating, suppressing the EOF to negligible values, which vary depending on the buffer pH and functional monomer included into the polymer chain.

Figure 3b provides a magnification of the two curves to highlight the slight difference in the EOF values when ethanolamine or amino-PEG are used to block the functional groups of poly(DMA-NAS-MAPS). The pH-dependent increase of the EOF is likely due to the presence of NAS moieties in the poly(DMA-NAS-MAPS) chains that did not quantitatively react with the blocking agent because of their inaccessible location on the polymer chain. The residual active esters of the polymer undergo hydrolysis and generate carboxyl groups, which become fully deprotonated under alkaline conditions. The reaction between amino PEG and NAS residues, although not quantitative, suppresses EOF more effectively. This might indicate that the negative charges, resulting from surface and polymer charges, are better shielded by the presence of grafted PEG chains even if their density is not very high.

3.5 Alkaline Protein Separation

1. Rinse the capillary with the running buffer by applying 20 psi for 3 min.
2. Inject hydrodynamically the sample mix (0.5 psi for 5 s).
3. Apply 30 kV at the cathode for 40 min.

Figures 4 and 5 show the electropherograms of the alkaline protein mixture. Efficient separations were observed, with low retention of proteins on the capillary walls. The stability of the coating is demonstrated by the profile of twenty run which shows no significant modification in the peak shape or retention time.

3.6 Acidic Protein Separation

1. Rinse the capillary with the running buffer by applying 20 psi for 3 min.

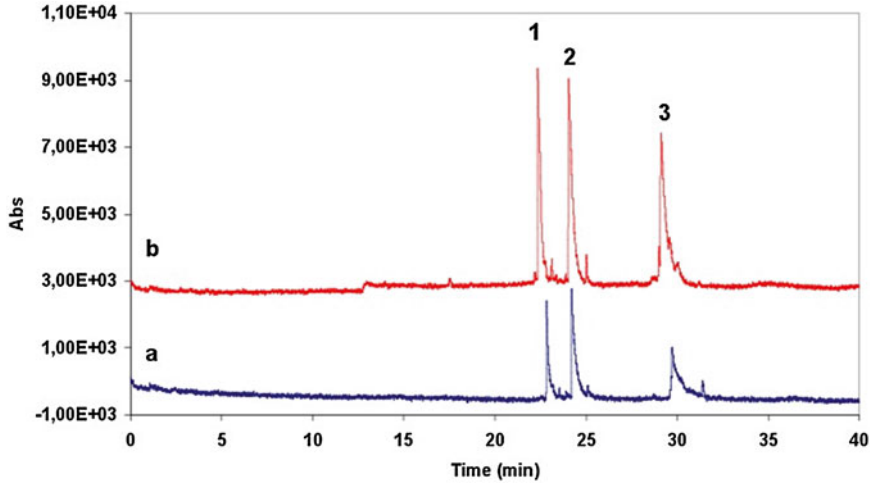

Fig. 4 Electropherograms of alkaline protein separation obtained using a capillary (30 μm ID, 110 cm total length, 100 cm to detection window) coated with poly(DMA-NAS-MAPS) and blocked with ethanolamine: (**a**) run #1, (**b**) run #20. In agreement with their pI values [3] the elution order is (1) lysozyme, (2) cytochrome C, (3) ribonuclease A. From Sola L. et al. [19] with permission

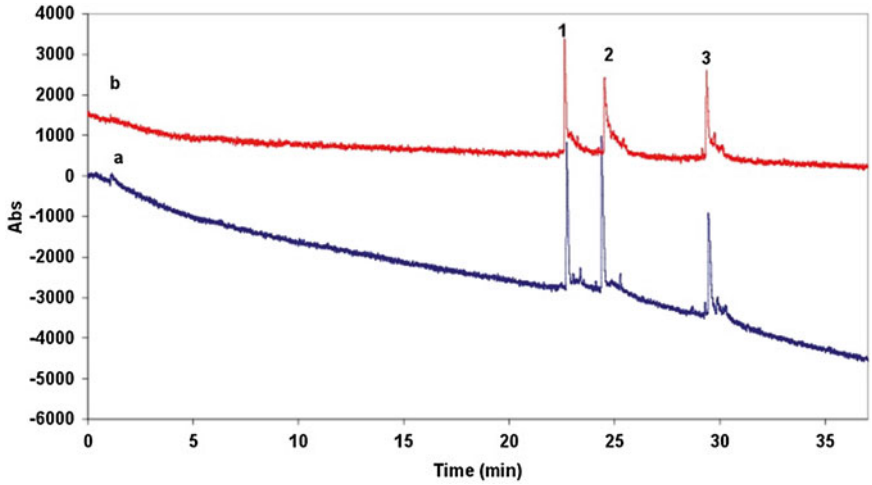

Fig. 5 Electropherograms of alkaline protein separation obtained using a capillary (30 μm ID, 110 cm total length, 100 cm to detection window) coated with poly(DMA-NAS-MAPS) and blocked with amino-PEG: (**a**) run #1, (**b**) run #20. In agreement with their pI values [3] the elution order is (1) lysozyme, (2) cytochrome C, (3) ribonuclease A. From Sola L. et al. [19] with permission

2. Inject electrokinetically the sample mix (10 kV for 5 s).
3. Apply −15 kV for 15 min.

Figure 6 represents the electropherogram of the acidic protein mixture (*see* **Note 16**). Twenty consecutive runs have been performed to demonstrate the good stability of the coating and the optimal charge shielding.

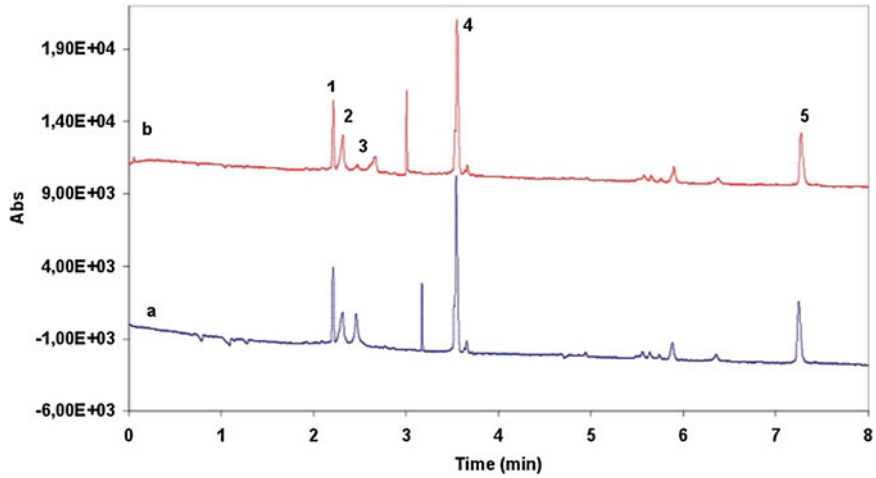

Fig. 6 Electropherograms of acidic protein separation obtained using a capillary (30 μm ID, 32 cm total length, 22 cm to detection window) coated with poly(DMA-GMA-MAPS): (**a**) run #1, (**b**) run #20. In agreement with their pI values [3] the elution order is (1) soy bean trypsin inhibitor, (2) β-lactoglobulin A and (3) B, (4) α-lactalbumin, (5) carbonic anhydrase. From Sola L. et al. [19] with permission

4 Notes

1. To apply positive pressure use nitrogen or compressed air as for a classic flash chromatography. A larger volume of DMA can be filtered and stored in a glass vial for no more than a week at 2–4 °C.
2. MAPS is an air-sensitive reagent. It is preferable to use a sealed bottle and take it using a syringe under inert atmosphere.
3. NAS is an air-sensitive reagent. Store it at 2–4 °C under inert and dry conditions. Prepare the needed quantity immediately before use, so to avoid prolonged exposure to air and humidity.
4. To avoid weighting small quantities of AIBN, a stock solution can be prepared as well. Prepare a 200 mM solution by dissolving AIBN into dry THF. Store it at −20 °C under inert atmosphere.
5. Prepare the coating solution immediately before the coating step. Do no store or recycle the coating solution.
6. The blocking solutions can be prepared in big quantities and stored in a dark place.
7. Use a needle to purge the gas directly into the reaction mixture, with a pressure of about 0.5 bar. The entire system has to be perfectly sealed, so regulate the gas stream to have the maximum flow without having gas leakage
8. Dissolve the solid reagents (such as NAS and AIBN) in a small quantity of anhydrous THF (1 mL) and add them using a syringe in order to keep the reaction environment dry and inert.

9. Fill a small balloon with helium and place it on top of the condenser, so to keep an inert atmosphere without the need of purging the reaction flask for 2 h.
10. At this step the system can be opened.
11. Use a pasteur pipette to drip the reaction mixture into the petroleum ether. Do not pour the entire reaction mixture all at once.
12. Considering the presence of active groups (NAS), filter it as soon as possible to avoid long exposure of the polymer to air and humidity.
13. To be sure the capillary is completely filled or rinsed, check with a litmus paper the pH of the liquid exiting the capillary.
14. Before use, store the capillary in dry environment.
15. After use, rinse the capillary with DI water, empty it completely and store in dry conditions at room temperature.
16. A further demonstration of the excellent EOF suppression capacity is obtained by the detection of the carbonic anhydrase peak, whose pI value is 6.2. Due to reduced charge, carbonic anhydrase has low mobility while migrating against the EOF toward the anode. Unless the EOF is completely suppressed it is difficult to detect this protein. The ability to observe this peak after so many consecutive runs demonstrates the stability of the coating on the capillary walls.

Acknowledgement

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References

1. Lookhart GL, Bean SR. (2004) Capillary electrophoresis of cereal proteins: an overview. J Capill Electrophor Microchip Technol 9:23–30
2. Lucy CA, MacDonald AM, Gulcev MD (2008) Non-covalent capillary coatings for protein separations in capillary electrophoresis. J Chromatogr A 1184:81–105
3. Lauer HH, McManigill D (1986) Capillary zone electrophoresis of proteins in untreated fused silica tubing. Anal Chem 58:166–170
4. McCormick RM (1988) Capillary zone electrophoretic separation of peptides and proteins using low pH buffers in modified silica capillaries. Anal Chem 60:2322–2328
5. Green JS, Jorgenson JW (1989) Minimizing adsorption of proteins on fused silica in capillary zone electrophoresis by the addition of alkali metal salts to the buffers. J Chromatogr A 478:63–70
6. Bachmann S, Vallant R, Bakry R et al (2010) CE coupled to MALDI with novel covalently coated capillaries. Electrophoresis 31: 618–629
7. Zhang Z, Yan B, Liu K et al (2009) CE-MS analysis of heroin and its basic impurities using a charged polymer-protected gold nanoparticle-coated capillary. Electrophoresis 30:379–387

8. Ullsten S, Zuberovic A, Wetterhall M et al (2004) A polyamine coating for enhanced capillary electrophoresis-electrospray ionization-mass spectrometry of proteins and peptides. Electrophoresis 25:2090–2099
9. Kašička V (2010) Recent advances in CE and CEC of peptides (2007-2009). Electrophoresis 31:122–146
10. Liu CY (2001) Stationary phases for capillary electrophoresis and capillary electrochromatography. Electrophoresis 22:612–628
11. Chiari M, Cretich M, Damin F et al (2000) New adsorbed coatings for capillary electrophoresis. Electrophoresis 21:909–916
12. Cretich M, Chiari M, Pirri G et al (2005) Electroosmotic flow suppression in capillary electrophoresis: chemisorption of trimethoxy silane-modified polydimethylacrylamide. Electrophoresis 26:1913–1919
13. Pirri G, Damin F, Chiari M et al (2004) Characterization of a polymeric adsorbed coating for DNA microarray glass slides. Anal Chem 76:1352–1358
14. Yalçin A, Damin F, Ozkumur E et al (2009) Direct observation of conformation of a polymeric coating with implications in microarray applications. Anal Chem 81:625–630
15. Cretich M, Reddington A, Monroe M et al (2011) Silicon biochips for dual label-free and fluorescence detection: application to protein microarray development. Biosens Bioelectron 26:3938–3943
16. Cretich M, Sedini V, Damin F et al (2008) Functionalization of poly(dimethylsiloxane) by chemisorption of copolymers: DNA microarrays for pathogen detection. Sens Actuators B 132:258–264
17. Cretich M, Sedini V, Damin F et al (2010) Coating of nitrocellulose for colorimetric DNA microarrays. Anal Biochem 397:84–88
18. Chiari M (2011) Patent application WO 2011124715 A1, silane copolymers and uses thereof, WO 2011124715 A1, 2011.
19. Sola L, Chiari M (2012) Modulation of electroosmotic flow in capillary electrophoresis using functional polymer coatings. J Chromatogr A 1270:324–329
20. Marangoni S, Rech I, Ghioni M et al (2010) A 6×8 photon-counting array detector system for fast and sensitive analysis of protein microarrays. Sens Actuators B 149:420–426
21. Luzinov I, Julthongpiput D, Malz H et al (2000) Polystyrene layers grafted to epoxy-modified silicon surfaces. Macromolecules 33:1043–1048
22. Williams BA, Vigh G (1996) Fast, accurate mobility determination method for capillary electrophoresis. Anal Chem 68:1174–1180
23. Mammen M, Dahmann G, Whitesides GM (1995) Effective inhibitors of hemagglutination by influenza virus synthesized from polymers having active ester groups. Insight into mechanism of inhibition. J Med Chem 38:4179–4190

Chapter 10

Measurement of Inflammatory Chemokines in Micro-dissected Tissue Biopsy Samples by Chip-Based Immunoaffinity Capillary Electrophoresis

Terry M. Phillips, Edward Wellner, Shane McMohan, and Heather Kalish

Abstract

To aid in the biochemical analysis of human skin biopsies, a chip-based immunoaffinity capillary electrophoresis (ICE) system has been developed for measuring inflammatory chemokines in micro-dissected areas of the biopsy. Following isolation of the areas of interest, the tissue was solubilized and the analytes of interest were isolated by the immunoaffinity disk within the chip. The captured analytes were labeled in situ with a 635 nm light-emitting laser dye and electro-eluted into the chip separation channel. Electrophoretic separation of all of the analytes was achieved in 2.5 min with quantification of each peak being performed by online LIF detection and integration of each peak area. The degree of accuracy and precision achieved by the chip-based system is comparable to conventional immunoassays and the system is robust enough to be applied to the analysis of clinical samples. Further, with the expanding array of antibodies that are commercially available, this chip-based system can be applied to a wide variety of different biomedical and clinical analyses.

Key words Capillary electrophoresis, Micro-dissection, Microchip, Immunoaffinity, Chemokines, Human biopsies

1 Introduction

Clinical inflammation is both a cellular and biochemical event [1, 2]. Cytokines and chemokines such as interleukin-1 beta (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-α) are the major players in the induction of fever and localized tissue damage. However, chemokines such as monocyte chemoattraction protein-1 (CCL2), macrophage inflammatory protein 3 beta (CCL19), secondary lymphoid tissue chemokine (CCL21), interleukin-8 (CXCL8), stromal cell-derived factor-1 (CXCL12), and B cell attracting chemokine-1 (CXCL13) are also important participants in the inflammatory process and mediators of cellular infiltration [3–8], leading to the onset of tissue injury [8]. Measurement of these chemokines is often difficult in plasma or

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serum due to their low circulating concentrations that bare little relevance to the events taking place at the site of injury [6, 7].

Immunocytochemistry has been applied to identifying chemokines within tissue biopsies and although this technique is a valuable tool in assessing the biochemistry of pathological lesions, it is usually difficult to quantify such components. There is a growing need for the development of a rapid procedure for assessing concentrations of inflammatory chemokines within the site of tissue injury, which could be used as a tool for assessing disease progression and for therapy evaluation.

Tissue biopsies are routinely performed in many disease states and the tissue obtained by procedures such as open wedge and needle biopsies provide suitable material for pathological assessment as well as providing materials for biochemical assessment. Frozen biopsy sections can be micro-dissected and dissected tissue from morphologically defined areas used for measurement of inflammatory factors [9]. Micro-dissection is often performed by laser capture micro-dissection on fixed routine pathological samples although manual micro-dissection of frozen tissue is preferable for analyzing molecules derived from the immune system [9]. In order to analyze such small samples, techniques such as capillary electrophoresis (CE) are often applied due to low sample requirement of 0.1 mL or less. CE also has the ability to measure multiple analytes within the same sample in a reasonably rapid manner. However, the speed of analysis slows considerably when CE is used for protein analysis of complex biological matrices such as tissue extracts. This problem can be overcome to a large extent by marrying CE with the selective characteristics of antibody capture or extraction prior to electrophoretic analysis.

Antibodies can act as pre-analysis concentrators thus improving the selectivity and resolving power of CE. Immunoaffinity CE (ICE) has been successfully applied to the analysis of a number of potential biomarkers in both human and animal biofluids [9–15]. The advantage of immunoaffinity CE over other immunoassays is that several analytes can be measured during the same run and the electrophoretic separation following the immunoaffinity extraction lessens the possibility of false positives [15]. The sensitivity of ICE can be further enhanced by the addition of laser-induced fluorescence (LIF) detection. Even with these modifications, immunoaffinity CE can become quite a lengthy and often difficult process thus lessening its advantages. This is true in the clinical arena where there is a need for rapid analyses that can be performed in relatively easy reach of the patient treatment room. The advent of microfluidic devices and their application to the development of chip-based CE addresses two important needs in clinical medicine: (a) the development of small instruments capable of processing extremely small samples, and (b) the capability to process samples in a reasonably quick and straightforward manner [6, 9–17]. In this chapter, we will detail the steps required to

micro-dissect human tissue sections and analyze the extracted materials for the presence of inflammatory chemokines using a chip-based CE system with a pre-separation antibody-based extraction port and LIF detection.

2 Materials

2.1 Buffers and Solutions

Prepare all solutions using ultrapure water (deionized water at an electrical resistance of 18 M Ω at 25 °C). Store all buffers at 4 °C unless indicated. Immediately prior to use, all solutions were passed through 0.2 mm NC filters to remove particulate impurities.

1. PO_4/Brij buffer: 100 mM phosphate, 0.1% v/v Brij 35, pH 7.4. Add 0.52 g monosodium phosphate monohydrate and 0.88 g anhydrous disodium phosphate to a 100 mL volumetric flask containing 30 mL distilled water. Mix well until the solids dissolve completely. Check the pH and adjust to 7.4 with 1 M HCl. Add 1 mL of Brij 35 detergent and add distilled water to the 100 mL mark. Store the solution at room temperature.
2. Tris buffer: 200 mM Tris–HCl, 9.0. Add 2.42 g of Tris base to 90 mL distilled water. Adjust the pH to 9.0 with 1 M HCl. Make up the final solution to 100 mL and store at room temperature.
3. ICE elution buffer: PO_4/Brij buffer buffered to pH 1.0 with 1 M HCl. Store at room temperature.
4. Tissue recovery buffer: Add 0.48 g of leupeptin to 10 mL PO_4/Brij buffer and buffer to pH 7.4 with 1 M NaOH. Store the solution at 4 °C and warm to 22 °C prior to use.
5. Sodium acetate buffer: 0.1 M sodium acetate, pH 5.0. Dissolve 1.36 g of sodium acetate trihydrate in 80 mL of distilled water. Add 0.6 mL of glacial acetic acid. Titrate the pH to 5.0 with glacial acetic acid and bring the total volume to 100 mL in a 100 mL volumetric flask. Store the solution at room temperature.
6. Sodium carbonate/bicarbonate buffer: 0.1 M bicarbonate, pH 9.0. Create solution A by dissolving 8.4 g of sodium bicarbonate in 100 mL of distilled water. Create solution B by dissolving 28.6 g of sodium carbonate in 100 mL of distilled water. These stock solutions can be stored at room temperature. Add 80 mL of solution A to 20 mL of solution B. Mix well and adjust the pH to 9.0 with 1 N hydrochloric acid. Store the final solution at room temperature.
7. Silane: 10% (v/v) aqueous solution. Dissolve 10 mL of 99% aqueous 3-aminopropyltriethoxy-silane in 90 mL water. Store the solution at 4 °C.

8. Sodium cyanoborate: Dissolve 1 mg of sodium cyanoborate in 1 mL distilled water and store at 4 °C and protect from light.
9. Cotton blue solution: Add 100 μL of aqueous cotton blue dye to 100 mL PO_4/Brij buffer. Store the solution at room temperature.
10. Sodium meta-periodate solution: Dissolve 0.214 g sodium meta-periodate in 10 mL distilled water and store at 4 °C.
11. Carbonyldiimazole solution: Dissolve 1 mg of carbonyldiimazole in 1 mL of formamide. Make this solution immediately before use.
12. Streptavidin solution: Dissolve 1 mg of streptavidin in 1 mL PO_4/Brij buffer. Store at 4 °C.
13. D-Biotin solution: Dissolve 1 mg of D-biotin in 1 mL PO_4/Brij buffer. Store at 4 °C.
14. Ethylene glycol: Heat 10 mL of distilled water to 37 °C. Add 1 g of 1500 Mw ethylene glycol and allow to completely dissolve. Cool to room temperature and store at that temperature.
15. AlexaFluor laser dye solution: Dissolve 5 μg of dry AlexaFluor 633 laser dye in 100 μL of formamide. Dissolve this solution in 900 μL of sodium bicarbonate and aliquot 20 μL of the dye solution into microcentrifuge tubes and store at –20 °C.
16. A set of standards are prepared by making dilutions of 10, 50, 100, 250, and 500 pg/mL for each chemokine by diluting the stock solution of each protein (1 μg/mL) in PO_4/Brij buffer. These standards should be stored at 4 °C for 1 week then discarded.

2.2 Reagents

1. Monocyte chemoattraction protein-1 (CCL2).
2. Macrophage inflammatory protein 3 beta (CCL19).
3. Secondary lymphoid tissue chemokine (CCL21).
4. Interleukin-8 (CXCL8).
5. Stromal cell-derived factor-1 (CXCL12).
6. B cell attracting chemokine-1 (CXCL13).
7. Anti-chemokine antibodies directed against chemokines (**items 1–6**). Both the chemokines and their respective antibodies were reconstituted to stock solutions of 1 μg/mL in PO_4/Brij buffer. The stock solutions should be divided into 1 mL aliquots and stored at –20 °C.
8. Long-chain hydrazine biotin.
9. 1 N hydrochloric acid.
10. Formamide.

2.3 Patient and Control Samples

Consent to use both patient and control samples was obtained from all subjects and no name indicators were assigned to any samples as required by the hospital institutional review board.

1. Routine skin biopsies from patients diagnosed with inflammatory allergic skin lesions at the George Washington University Hospital, Washington, DC, USA.
2. Routine skin biopsies from non-allergic, normal subjects age-matched to the patient groups.

2.4 Instruments and Equipment

1. Micralyne μTK microfluidic electrophoresis system (Micralyne, Edmonton, Alberta, Canada—Fig. 1) equipped with four dual-channel 6 kV power boards, eight platinum electrodes, a chip stage form-fitted to accept 16-mm × 95-mm × 2.2-mm deep CE chips and a 8-μW 635 nm red diode laser. Detection was achieved using an epi-illumination microscope coupled with a Hamamatsu H5773-03 photomultiplier tube and a 16-bit data acquisition board.
2. PC computer running Windows and a compiled LabView interface for controlling the μTK system, the syringe pumps and collecting data from the detector acquisition board.
3. 2 Microsyringe pumps.
4. Low-fluorescence "Borofloat" glass CE chips (Micralyne) in a custom format designed by us within the manufacturer's guidelines (Fig. 2). All of the chip channels were 20-μm deep × 50-μm wide semicircular channels ending in a 2.0-mm diameter × 0.1-mm deep port. Separation was achieved using the 120-mm (115-mm to the detector) serpentine channel running between ports 5 and 6, which acted as the main CE buffer reservoirs.
5. Eppendorf Micro-dissector equipped with an oscillating micro-chisel, two manipulation joysticks, a micro-pipette, and a cooling stage (*see* **Notes 2** and **3**) (Fig. 3).

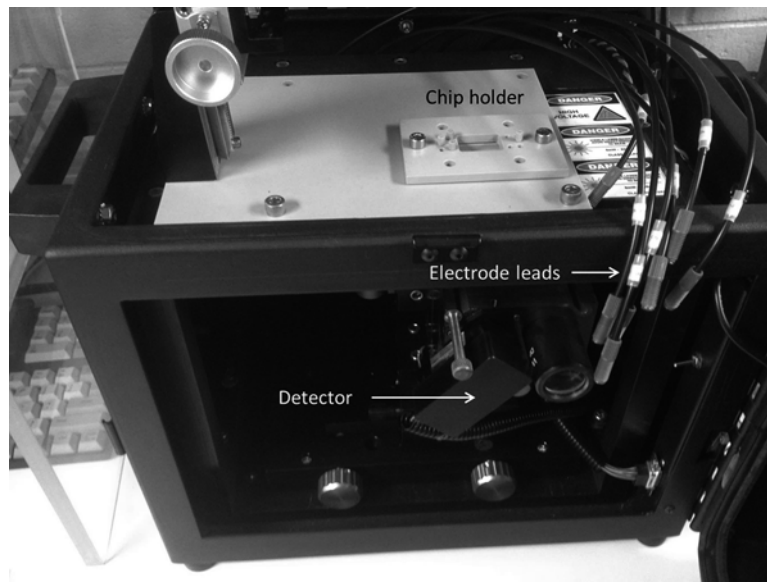

Fig. 1 The microlyne μTK CE system

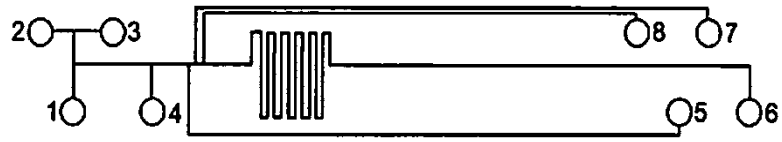

Fig. 2 Diagrammatic representation of the custom-designed ICE chip: the numbers correspond to the port numbering system employed in the text of Subheading 3.4. Reproduced from ref. [8] with permission from John Wiley & Sons

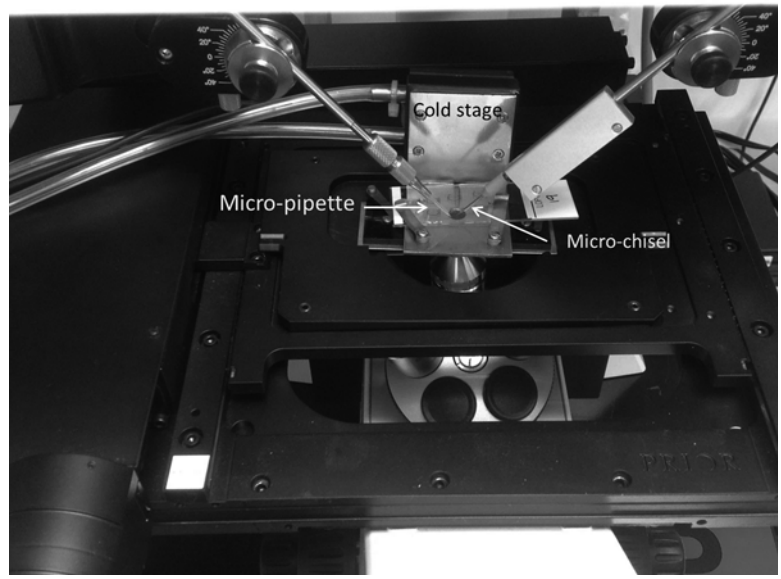

Fig. 3 Micro-dissector system

6. NanoDrop ND-100 micro-spectrophotometer.
7. 13 mm diameter, 0.2-μm polyethersulfone membrane filters.
8. Poly-ether-ether-ketone (PEEK) tubing (50-μm id, 360-μm OD).
9. 100 kDa cut-off ultramicro Spin Con dialyzer/concentrators.
10. Upchurch Scientific N-125H type NanoPort assemblies.
11. AP40 glass fiber filters.
12. Upchurch Scientific F-126Hx type nuts (*see* **Note 1**).
13. Upchurch Scientific P554 type plugs.

3 Methods

3.1 Biotinylation of the Anti-chemokine Antibodies

1. Suspend 1 μg of antibody in 100 μL of sodium acetate buffer (*see* **Note 3**).
2. Cool to 4 °C in an ice bath.

3. Add 100 μL of ice-cold sodium metaperiodate (*see* Subheading 2.1).
4. Mix and place on a rotary mixer for 20 min at 4 °C in the dark (*see* **Note 4**).
5. Stop the reaction by adding 100 μL of ethylene glycol (*see* **Note 5**).
6. Dialyze for 5 h against PO_4/Brij at 4 °C in the dark.
7. Remove the antibody and place in a stoppered glass tube.
8. Add 50 μL of sodium cyanoborate.
9. Add 1 μg of long-chain hydrazine-biotin.
10. Mix and place on rotary mixer for 60 min at room temperature.
11. Dialyze against PO_4/Brij overnight at 4 °C.
12. Store at 4 °C until required.

3.2 Preparation of the Immunoaffinity Disks

1. Punch 2.0-mm diameter disks from the AP40 glass fiber filters (*see* **Note 6**).
2. Place disks into a glass petri dish containing 5 mL of silane at 100 °C for 60 min.
3. Cool to 22 °C, drain by suction and placed into a fresh 5 mL of silane.
4. Repeat **steps 2–3** four times before draining the dish and adding 5 mL of hydrochloric acid.
5. Incubate the disks for 60 min at 100 °C.
6. Cool to 22 °C, drain by suction and wash 2× in 10 mL distilled water.
7. Place the disks into a clean glass petri dish containing 5 mL of carbonyldiimazole (*see* **Note 7**).
8. Incubate for 6 h at 22 °C on a rotary shaker.
9. Drain by suction and wash the disks 5× in 5 mL formamide.
10. Remove the disks and air-dry at room temperature.
11. Place the dry disks into a glass petri dish and add 5 mL of streptavidin solution.
12. Incubate for 12 h at 22 °C on a rotary plate mixer.
13. Drain by suction and wash 5× in 10 mL of PO_4/Brij buffer (*see* **Note 8**).
14. Drain the petri dish by suction and add 5 mL of a solution containing a 10 ng mixture of each biotinylated antibody (*see* **Note 9**).
15. Incubate in a petri dish with moist filter paper for 1 h.
16. Drain and wash the disks 5× in 5 mL PO_4/Brij buffer.

17. Drain the disks and add 10 mL of D-biotin solution and incubate for 30 min at 22 °C (*see* **Note 10**).
18. Finally, drain the petri dish and wash the disks twice in a 10 mL solution of PO_4/Brij buffer.
19. Transfer to a small beaker and seal with parafilm.
20. Store the finished immunoaffinity disks in a dry, sealed petri dish at 4 °C until required.

3.3 Micro-dissection of Tissue Samples

1. Snap-freeze the tissue samples in liquid nitrogen (*see* **Note 11**).
2. Using a cryostat cut two 10-μm thick frozen sections from each sample block.
3. Place onto glass microscope slides previously chilled to 4 °C.
4. Air-dry the sections at room temperature and stain with cotton blue (*see* **Note 12**).
5. Place the stained section under the micro-dissector and using the inverted microscope select areas containing either lymphoid cell infiltration or normal tissue (*see* **Note 13**).
6. Bring the oscillating micro-chisel into focus and touch the tissue section (Figs. 4 and 5).
7. Using the micro-dissector joystick, gently move the tip of the micro-chisel around the area of interest within the tissue section, thus creating a tissue "island" (*see* **Note 14**).
8. Bring the micro-pipette into focus and using the other joystick to center the micropipette tip over the island.
9. Flood the island with 22 μL of tissue recovery buffer (*see* **Note 15**).

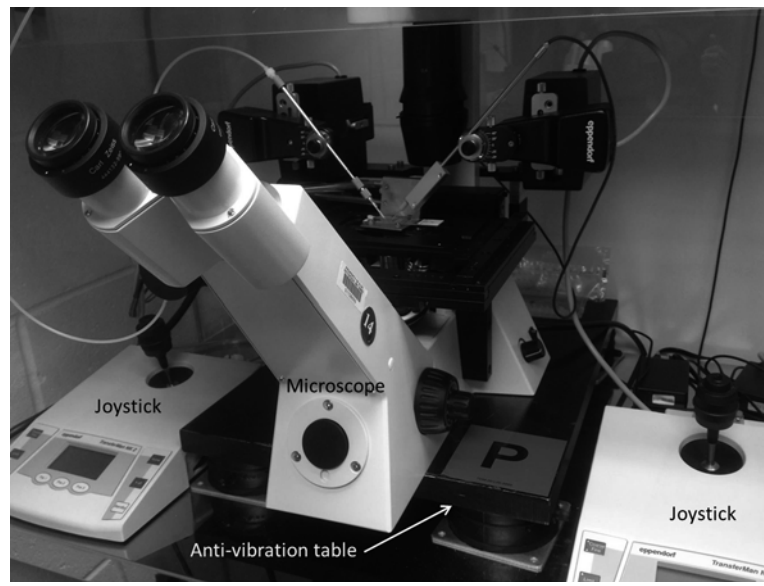

Fig. 4 Micro-dissector mounted on an inverted microscope

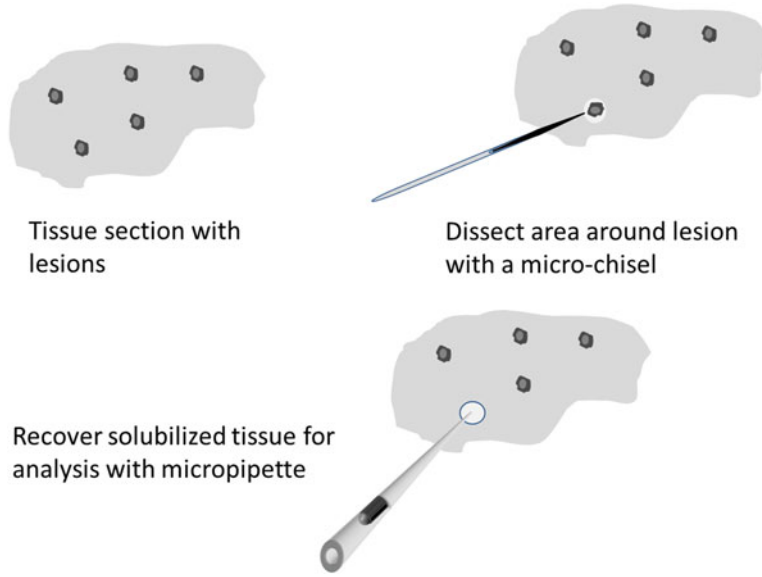

Fig. 5 Schematic of the micro-dissection procedure showing the main steps employed in the procedure as described in Subheading 3.4

10. Incubate for 2 min then recover the fluid plus the cellular debris using the same pipette and transfer to a Spin Con concentrator (*see* **Note 16**).
11. Spin the dialyzer at 10,000 × *g* for 5 min (*see* **Note 17**).
12. Measure the total protein content of the clarified fluid using a Nanodrop microspectrophotometer (*see* **Note 18**).
13. Adjust the protein concentration of each sample to 1 μg/mL in PO_4/Brij buffer.
14. Store at 4 °C until analysis.

3.4 ICE Measurement of Chemokines in Tissue Sections

1. Using a clean chip place a Nanoport to each of the eight chip ports (*see* **Note 19**).
2. Gently place an immunoaffinity disk into port 1 on the chip.
3. Using a modified F-126x nut attach a length of PEEK tubing to Nanoport 1.
4. Using an unmodified F-126X nut attach a length of PEEK tubing to Nanoport 2 and 7.
5. Place the tubing from Nanoport 2 into a waste container.
6. Place modified P554 plugs into Nanoports 3–6.
7. Connect the tubing from Nanoport 1 to pump 1.
8. Connect the tubing from Nanoport 7 to pump 2.
9. Connect each electrode wire to an electrical outlet on the μTK CE unit (*see* **Note 20**).

10. Program the Labview interface (*see* **Note 21**).
11. Using pump 2 set at a pump speed of 2 mL/min prime the entire system with PO_4/Brij buffer.
12. Shut off pump 2 and fill Nanoport 3 with AlexaFluor laser dye and Nanoport 4 with ICE elution buffer.
13. Program pump 1 to introduce a 200 nL sample into Nanoport 1.
14. Incubate the sample with the immunoaffinity disk for 4 min in Nanoport 1 (*see* **Note 22**).
15. Program pump 1 to flush 0.5 mL of PO_4/Brij buffer from Nanoport 1 into Nanoport 2 (*see* **Note 23**).
16. Program the electrodes in Nanoport 3 and 4 to apply a 4 kV potential from Nanoport 3 to Nanoport 1 for 30 s while keeping Nanoport 4, 5, 6, 7, and 8 at ground, to electrokinetically move the AlexaFluor laser dye from Nanoport 3 into Nanoport 1 (*see* **Note 24**).
17. Switch off the electricity and allow the dye to react with the bound analytes for 2 min (*see* **Note 25**).
18. Program pump 1 to flush 0.5 mL of PO_4/Brij buffer through Nanoport 1 to Nanoport 2 (*see* **Note 26**).
19. Release the bound analytes by programming a 6 kV potential between Nanoport 4 and Nanoport 1 for 45 s to electrokinetically move the ICE elution buffer into Nanoport 1: maintain all of the other electrodes at ground.
20. Shut off the electricity and allow the ICE elution buffer to remain in Nanoport 1 for 2 min (*see* **Note 27**).
21. Program a 6 kV potential between Nanoport electrodes 1 and 5 for 25 s to move the released analytes into the separation channel.
22. Program a 6 kV potential between Nanoport 5 and 6 for 3 min (*see* **Note 28**).
23. Online detection is achieved by placing the detector at a point 1 cm from Nanoport 6.
24. Using pump 2, flush the entire system for 1 min before performing the next analysis. Figure 6 is a diagrammatic representation of this process.

3.5 Calculating Chemokine Values

1. Construct calibration curves by running a set of known chemokine standards through the ICE system (figure 7).
2. Using the peak areas, calculate the concentration of each individual chemokine against these curves (*see* **Note 29**).
3. Figure 8 illustrates the different electropherograms produced from (a) tissue extracted from an atopic patient lesion and (b) norm tissue extract submitted to the ICE analysis. Note the different y-axis values in the two samples.

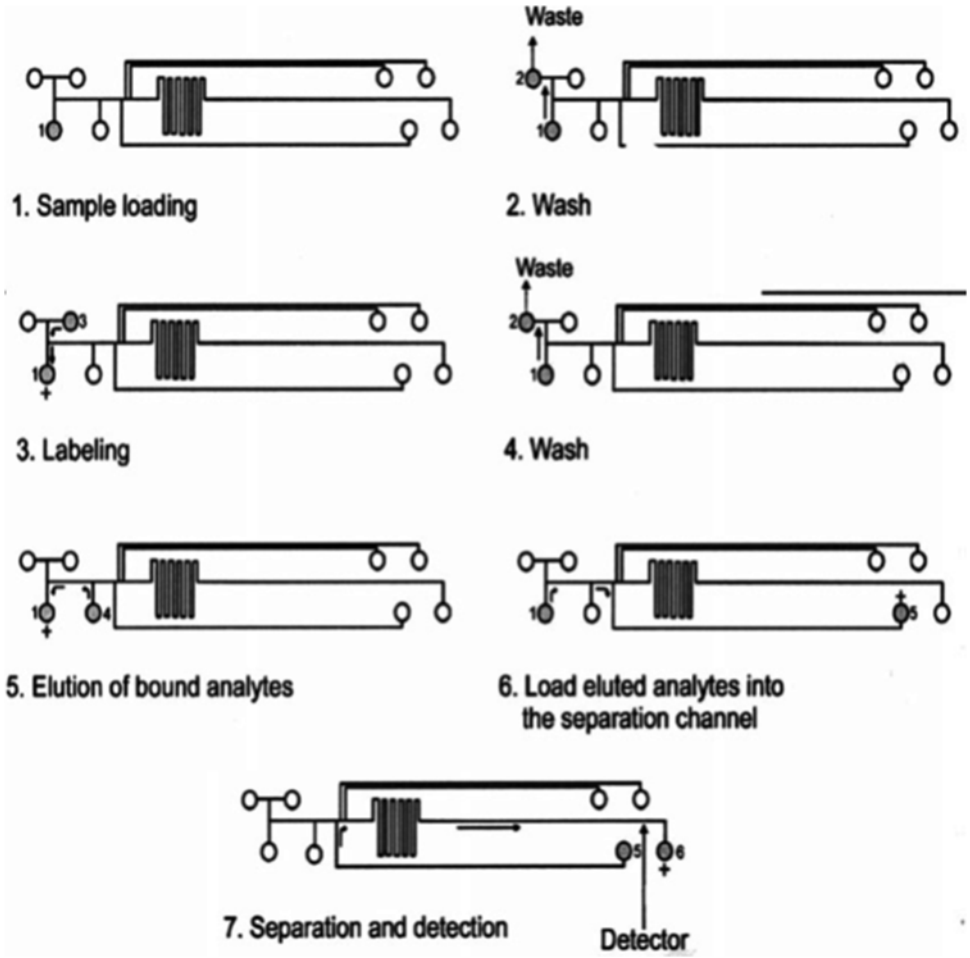

Fig. 6 Diagrammatic showing the procedural steps performed during ICE isolation of chemokines from microdissected tissue. Step 1, the sample is injected into port 1. Step 2, following analyte capture, nonreactive materials are flushed from port 1 to port 2. Step 3, laser dye is moved from port 3 into port 1 where it reacts with the bound analytes. Step 4, free dye is flushed to port 2. Step 5, recovery of the bound analytes is achieved by moving a plug of elution buffer from port 4 into port 1. Step 6, the eluted analytes are removed from port 1 and migrated into the separation channel between ports 1 and 5. Step 7, the labeled analytes are separated and detected online by an electrical current between ports 5 and 6. Reproduced from ref. [8] with permission from John Wiley & Sons

4 Notes

1. The standard F-125H nut (available from Upchurch Scientific, Oak Harbor, WA, USA) is modified by cutting a groove down the length of the outside of the nut and attaching a thin platinum wire electrode into the groove with methacrylate glue. The electrode is flattened at the bottom to fit into the chip port. The pitch of the nut is then reshaped with a screw

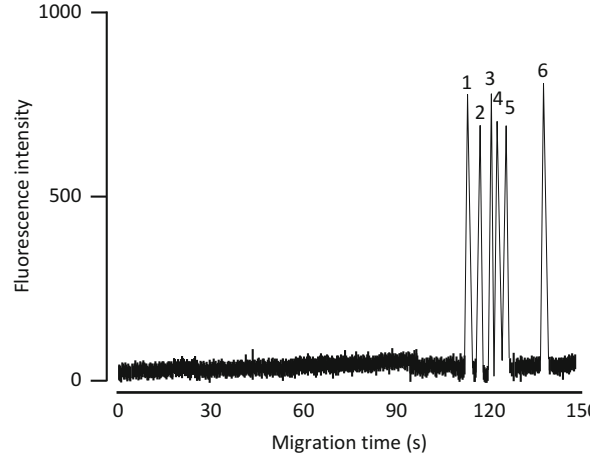

Fig. 7 Immunoaffinity electropherogram of a mixture containing 100 pg/mL of each chemokine standard recovered by the ICE system. Conditions as described in section 3.4. Peaks = 1: CXCL8, 2: CXCL12. 3: CCL2, 4:CXCL13, 5: CCL19, 6: CCL21

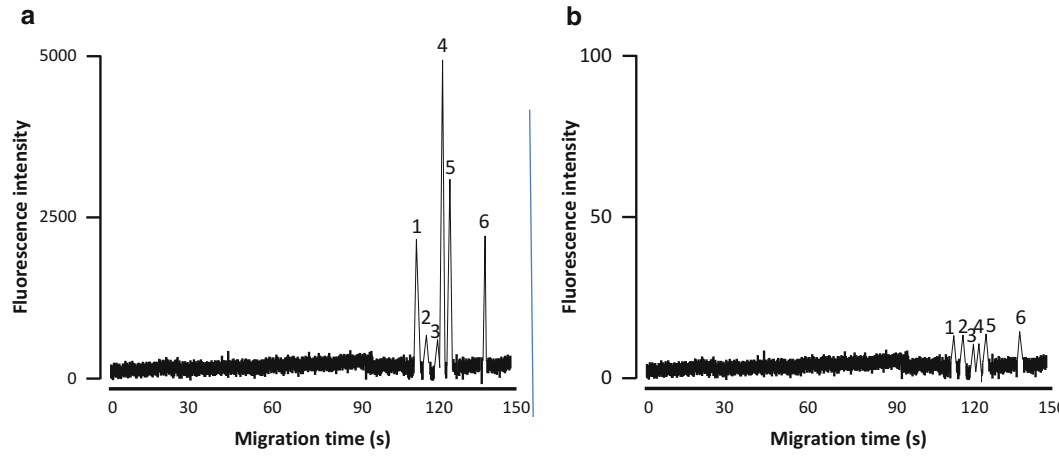

Fig. 8 Immunoaffinity electropherogram of chemokines isolated from micro-dissected tissue lesions in **a**) a patient with inflammatory atopic dermatitis and **b**) normal skin. Peaks = 1: CXCL8, 2: CXCL12. 3: CCL2, 4: CXCL13, 5: CCL19, 6: CCL21.

cutting dye. These modified nuts were used for both fluidic transfers and electrophoresis.

2. The micro-dissector assembly is attached to a standard inverted microscope using the clamping device supplied with the micro-dissector (Fig. 4).
3. Antibodies directed against each individual chemokine were biotinylated via their carbohydrate moieties using the technique

of O'Shannessy and Quarlesl [18] prior to immobilization onto the activated immunoaffinity disk surface.

4. The antibodies and the mixer were placed in a refrigerator to maintain both temperature and dark conditions.
5. It was found that 1500 Mw ethylene glycol was the most effective and could easily be removed by dialysis.
6. The 2-mm diameter disks were punched out using a laboratory-built hollow steel punch with a sharpened cutting end. The punch was struck twice with a small hammer and the disk recovered by pushing it out of the punch with a rod.
7. A 1 mg of carbonyl diimazole in 1 mL formamide solution must be made up immediately before use as the reagent absorbs moisture from the air and becomes inactive.
8. Block the non-reacted carbonyl diimazole side chains by incubation in 5 mL of a 200 mM Tris–HCl buffer, pH 9.0 for 30 min.
9. As the in situ concentrations of the chemokines are unknown, the immunoextraction disk was loaded with approximately 50-fold excess of each antibody; a concentration previously found to be efficient for adequate analyte capture [8]. The disk contained a mixture of immobilized antibodies directed against the individual chemokines and manufactured as previously described [19–21].
10. Block any excess biotin binding sites on the immobilized streptavidin
11. Frozen sections were used as previous experience had shown that formalin fixed materials were unsuitable for the recovery of soluble proteins and peptides from micro-dissected tissue [10, 20].
12. Application of the dye aids in morphological identification of the tissue, especially the lesion areas which have infiltrating leukocytes.
13. Atopic skin lesions contain perivascular lesions with clusters of immune cells surrounding a small blood vessel staining as small blue clusters as opposed the normal non-affected skin tissue.
14. The oscillating microchisel will cut away or remove the tissue surrounding the area of interest, thus leaving an "island" of tissue surrounded by clear glass.
15. The micropipette is operated by a Eppendorf CellTram air manual piston pump. Fluid is drawn into the micropipette by twisting the top of the CellTram. A quarter turn of the top will draw up 22 μL of fluid. Reversing the turn will eject the fluid from the micro-pipette.
16. The concentrator has a sleeve containing the specific molecule weight cut-off filter (10,000 kDa) and a collection tub.

The recovered fluid is placed into the sleeve, which in turn is placed into the tube.

17. This removes the extraneous macromolecules and cellular debris prior to analysis.
18. Total protein content of each clarified sample was measured by direct spectrophotometry at 280/260 nm using the NanoDrop microspectrophotometer and adjusted to a protein concentration of 1 μg/mL in the PO4/Brij buffer [22].
19. To mount the Nanoport on the chip port, one has to attach the adhesive ring (that comes with the Nanoport assembly kit) to the top of the chip, directly over the port. Then carefully place the Nanoport over the chip port and press down firmly. Hold the Nanoport onto the chip by placing a strong binder clip over both pieces and place the whole into a 100 °C oven for 2 h. Cool to room temperature before removing the binder clip.
20. The μTK CE unit has eight electrode connections at the back of the instrument. The company provides a set of eight electrode connection leads, each lead being numbered to a specific electrode. The platinum wires, from the modified F126 nuts, are attached to the appropriate lead.
21. The entire procedure, including sample injection, immunoaffinity isolation, and analyte labeling, together with separation and analysis was programmed into the LabView interface sequence, which then controlled the injection pump, the flush pump, and the CE system.
22. Four minutes was found experimentally to be the ideal time for optimal antibody–antigen reactions to take place using anti-chemokine antibodies from R & D Systems.
23. This step washes away all unbound materials.
24. AlexaFluor 633 is a dye excited at 633 nm and optimally suited to the output of the laser used in the electrophoresis system.
25. The 2 min incubation was found experimentally to be the ideal time for optimal analyte labeling in this system. Experimentation showed that at 2 min the dye consistently bound to 89 % of the available protein.
26. This step removes all of the unreacted dye from the immunoaffinity port prior to elution, separation, and detection of the captured chemokines.
27. The elution buffer was allowed to interact with the immobilized immune complex for 2 min, during which time the acidic buffer disrupted the FAb/analyte complex thus releasing the bound analyte for analysis by electrophoresis. The incubation time was determined experimentally by electrophoresis. This incubation time was determined experimentally by spectrophotometrically measuring the amount of analyte released from an immunoaffinity disk over time.

28. This drives the analytes down the separation channel thus separating the different chemokines prior to on-line detection Fig. 8.
29. The concentrations of the separated chemokines are calculated by comparing the peak areas of the sample analytes to peak area calibration curves constructed by running known amounts of each chemokine through the system.

Acknowledgement

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References

1. Medzhitov R (2008) Origin and physiological roles of inflammation. Nature 454:428–435
2. Nedoszytko B, Sokołowska-Wojdyło M, Ruckemann-Dziurdzińska K et al (2014) Chemokines and cytokines network in the pathogenesis of the inflammatory skin diseases: atopic dermatitis, psoriasis and skin mastocytosis. Post Dermatol Alergol 31:84–91
3. Brandes M, Legler DF, Spoerri B et al (2000) Activation-dependent modulation of B lymphocyte migration to chemokines. Int Immunol 12:1285–1292
4. Collington SJ, Hallgren J, Pease JE et al (2010) The role of the CCL2/CCR2 axis in mouse mast cell migration in vitro and in vivo. J Immunol 184:6114–6123
5. Castro PR, Marques SM, Viana CT et al (2014) Deletion of the chemokine receptor CCR2 attenuates foreign body reaction to implants in mice. Microvasc Res 95:37–45
6. Turner MD, Nedjai B, Hurst T et al (2014) Cytokines and chemokines: at the crossroads of cell signalling and inflammatory disease. Biochim Biophys Acta 1843:2563–2582
7. Kalish H, Phillips TM (2009) The application of micro-analytical techniques to biomedical analysis. Curr Pharm Anal 5:208–228
8. Patadia M, Dixon J, Conley D et al (2010) Evaluation of the presence of B-cell attractant chemokines in chronic rhinosinusitis. Am J Rhinol Allergy 24:11–16
9. Kramer JM, Klimatcheva E, Rothstein TL (2013) CXCL13 is elevated in Sjögren's syndrome in mice and humans and is implicated in disease pathogenesis. J Leukoc Biol 94:1079–1089
10. Phillips TM, Wellner EF (2007) Analysis of inflammatory biomarkers from tissue biopsies by chip-based immunoaffinity CE. Electrophoresis 28:3041–3048
11. Wellner EF, Kalish H (2008) A chip-based immunoaffinity capillary electrophoresis assay for assessing hormones in human biological fluids. Electrophoresis 29:3477–3483
12. Phillips TM, Kalish H, Wellner E (2009) Receptor affinity CE for measuring bioactive Inflammatory cytokines in human skin biopsies. Electrophoresis 30:3947–3954
13. Phillips TM, Wellner EF (2009) Chip-based immunoaffinity CE: application to the measurement of brain-derived neurotrophic factor in skin biopsies. Electrophoresis 30: 2307–2312
14. Kalish H, Phillips TM (2010) Analysis of neurotrophins by immunoaffinity capillary electrophoresis following traumatic head injury. J Chrom B 878:194–200
15. Kalish H, Phillips TM (2012) Assessment of chemokine profiles in human skin biopsies by an immunoaffinity capillary electrophoresis chip. Methods 56:198–203
16. Oskvig DB, Elkahloun AG, Johnson KR (2012) Maternal immune activation by LPS selectively alters specific gene expression profiles of interneuron migration and oxidative stress in the fetus without triggering a fetal immune response. Brain Behav Immunol 26:623–634
17. Guzman NA, Phillips TM (2011) Immunoaffinity capillary electrophoresis: a new versatile tool for determining protein biomarkers in inflammatory processes. Electrophoresis 32:1565–1578
18. O'Shannessy DJ, Quarles RH (1987) Labeling of the oligosaccharide moieties of immunoglobulins. J Immunol Methods 99:153–161
19. Phillips TM (2004) Rapid analysis of inflammatory cytokines in cerebrospinal fluid using chip-based immunoaffinity electrophoresis. Electrophoresis 25:1652–1659

20. Phillips TM, Wellner E (2006) Measurement of neuropeptides in clinical samples using chip-based immunoaffinity capillary electrophoresis. J Chromatogr A 1111: 106–111
21. Phillips TM, Wellner E (2013) Detection of cerebral spinal fluid-associated chemokines in birth traumatized premature babies by chip-based immunoaffinity CE. Electrophoresis 34:1530–1538
22. Pierce Biotechnology Technical Tip #6 (2008) Extinction coefficients. http://www.piercenet.com/previews/guides/tech-tips/ Accessed 10 July 2008

Chapter 11

Separation of Recombinant Therapeutic Proteins Using Capillary Gel Electrophoresis and Capillary Isoelectric Focusing

Caitlyn A.G. De Jong, Jessica Risley, Alexis K. Lee, Shuai Sherry Zhao, and David D.Y. Chen

Abstract

Detailed step-by-step methods for protein separation techniques based on capillary electrophoresis (CE) are described in this chapter. Focus is placed on two techniques, capillary gel electrophoresis (CGE) and capillary isoelectric focusing (cIEF). CGE is essentially gel electrophoresis, performed in a capillary, where a hydrogel is used as a sieving matrix to separate proteins or peptides based on size. cIEF separates proteins or peptides based on their isoelectric point (pI), the pH at which the protein or peptide bears no charges. Detailed protocols and steps (including capillary preparation, sample preparation, CE separation conditions, and detection) for both CGE and cIEF presented so that readers can follow the described methods in their own labs.

Key words Capillary isoelectric focusing, Capillary gel electrophoresis, Recombinant therapeutic proteins, Protein characterization

1 Introduction

The production of recombinant proteins has led to important advancements in biotherapeutics for medical treatment. To date, there are more than 200 protein-based drugs commercially available and many are used to treat specific diseases [1]. Recombinantly produced proteins have several therapeutic advantages to nonrecombinant proteins. This includes the ability to be produced more efficiently and inexpensively using hosts such as bacterial or other easy-to-maintain cell lines. Also, the use of recombinant protein technology allows for modification of protein, which can often improve drug efficacy or specificity [2].

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Several techniques exist to analyze recombinant proteins, such as gel electrophoresis, Western blotting [3], precipitation [4], centrifugation [4], and various types of chromatographic methods [5, 6] (e.g., ion exchange, affinity). Most of the currently used techniques have room for improvement to overcome various drawbacks, including the lack of selectivity, and time-consuming and/or labor intensive. Capillary electrophoresis (CE) can be effectively used to address some of these concerns.

CE separations are in many cases faster than most other chromatography or electrophoresis processes while using very small amounts of sample [7]. The basis of this technique is the application of a high voltage across a capillary filled with a background electrolyte (BGE). Charged species will move in the electric field within the capillary according to their own charge to size ratios, and can thus be separated. CE is also a versatile tool that can be tailored for different purposes by simply modifying its inner wall properties or electrolyte composition.

One of the frequently used CE techniques for protein separations is capillary gel electrophoresis (CGE). Traditional polyacrylamide slab gel electrophoresis has been used in biochemical analysis for decades, and CGE is based on the same principle by performing the separation in a gel-filled capillary instead [8]. Slab gel electrophoresis uses a gel matrix which contains pores and acts as a sieve to separate proteins based on their size as they migrate through the gel, and tends to be both time-consuming and labor intensive. CGE maintains separation efficiency by separating analytes in a viscous linear polymer solution (typically 4–10% w/v, although up to 20% may be used) that is often replaceable [9, 10]. An automated instrument is used, along with on-column detection to provide accurate quantification on small volumes of sample. While (poly)acrylamide gels (non-cross-linked or cross-linked) tend to be the most common, there are many variants of this technique using different gel matrices, such as poly(dimethylacrylamide), poly(ethylene oxide), and hydroxypropylmethyl cellulose, as well as other less commonly used gels [11].

Another important CE technique for protein separation is capillary isoelectric focusing (cIEF). cIEF separates proteins by their pI by generating through the use of ampholytes, a pH gradient across the capillary, and allowing proteins to move under an applied voltage until their net charge becomes zero. At this stage, the analytes focus into thin bands. In this way it allows for the separation of amphoteric compounds that have different isoelectric points.

In cIEF separations it has been shown that by adding sacrificial ampholytes to the mixture of carrier ampholytes (CAs) and sample, the problem of sample loss from the capillary is mitigated [12]. Also, during the focusing step sacrificial ampholytes help ensure detection, since for detection of sample and CAs they need to be confined between the inlet (anode) and the detection window of the capillary [13].

For cIEF detection there are two options: either whole column detection or single-point detection. Single-point detection works like typical CE UV detection, where analytes move past the detector. In order to mobilize the proteins which have focused at their pIs in cIEF, hydrodynamic or chemical mobilization is used. Hydrodynamic mobilization relies on an applied pressure to cause proteins to migrate towards the detector, while chemical mobilization relies on a change in the chemical environment to cause protein migration. Whole column detection avoids resolution problems associated with the mobilization process in conventional cIEF [14], and is analogous to traditional detection for slab gel IEF. For whole column detection, the separation capillary is typically 50 mm in length, and all of the focused sample zones within the capillary are detected simultaneously. However, to perform whole column imaging, a specific detection instrument is required. It is worth noting that cIEF can also be performed with Mass Spectrometry as a detector [15], but this will not be discussed in the following methods because it poses a different set of challenges compared to optical detection. In the method for cIEF that we present, optical point detection will be used because it is the most accessible mode of detection for all CE instruments.

The main steps of the methods that will be outlined in the Materials and Methods section of this chapter include solution preparation, capillary preparation, sample preparation, separation conditions, and detection. Methods will be described in detail including filling a capillary with a gel matrix, or coating the inside of the capillary, preparing protein samples specifically for injection into the CE for each method, as well as how to set up the CE method program and which running conditions to use, and the detection method details.

2 Materials

2.1 Instrumentation and Equipment

- CE instrument.
- Capillaries (50, 75, or 100 μm internal diameter (I.D.)) (*see* **Note 1**) and cartridge.
- Detector (Ultraviolet (UV) or Photodiode Array (PDA)).
- Water aspirator system for degassing samples and BGE (filter flask, rubber stopper, vacuum tubing, tap adapter).
- Benchtop centrifuge.
- Microcon-10 kDa Centrifugal Filter Unit with Ultracel-10 membrane (Millipore), membranes of different sizes may be used depending on the protein(s) present in the sample.
- Freezer set to −20 °C.

2.2 Solutions and Standards

Method 1—CGE [16]

- Diluted NaOH solution: 0.01 M NaOH in deionized water.
- Gel solution: place 1.88 g acrylamide in 15 mL deionized water with 19 mg (24.52 μL) *N,N,N′,N′*-tetramethylethylenediamine (TEMED), degas for 10 min by water aspiration, and then add 23 mg ammonium persulfate (APS) in 10 mL of deionized water (degas final solution again for 10–15 min).
- Phosphate buffer: 0.05 M phosphoric acid solution in deionized water adjusted to pH 5.5 by NaOH.
- SDS buffer: 0.5% w/v solid sodium dodecyl sulfate (SDS) in phosphate buffer (described above).
- Background electrolyte solution: dissolve 2 g (approximately 2 mL) of gel solution in 20 mL of SDS solution (described above) to obtain 10% w/v of gel in the run buffer.
- Sample solution: dissolve 0.5–1.5 mg of standard or sample protein(s) in 1 mL of SDS buffer (described above).

Method 2—CGE [17]

- Diluted NaOH solution: 0.01 M NaOH in deionized water.
- Acrylamide solution: prepare 6% w/v acrylamide in 5 mL of deionized water, degas for 30 min by water aspiration, and then add 100 μL of 10% APS, 20 μL of 10% TEMED, and 50 μL of 10% SDS and stir gently to mix.
- Tris-borate buffer: 0.1 M Tris–HCl and 0.25 M borate (using sodium tetraborate) (pH 8.1) in deionized water.
- Sample solution: dissolve 0.5–1.0 mg protein standards/samples in 1 mL Tris-borate buffer (described above) and SDS in a ratio of 2.5:1 w/w SDS:protein (e.g., if 0.5 mg protein used, use 1.25 mg SDS).

Method 3—Capillary Isoelectric Focusing (cIEF) [13]

- Carrier Ampholytes: Pharmalyte p*I* 3–10 CAs (PN 17-0456-01 from GE Healthcare Life Sciences).
- Anolyte solution: 200 mM H_3PO_4 in deionized water.
- Catholyte solution: 300 mM NaOH in deionized water.
- Chemical Mobilizer solution: 350 mM acetic acid in deionized water.
- Capillary Cleaning solution: 4.3 M urea in deionized water.
- Urea containing CIEF polymer solution: Dissolve urea to a final concentration of 3 M into Beckman Coulter's/AB SCIEX's CIEF polymer solution, and degas at 2000 G-force with a centrifuge.

- Buffer Replacement solution: 20 mM Tris–HCl (pH 8) in deionized water.
- Sacrificial ampholytes (anodic and cathodic blockers). Recommended: iminodiacetic acid (IMD) and L-Arginine (ARG) (*see* **Note 2**).
- cIEF Master Mix: 50% v/v cIEF polymer solution in deionized water with 2% w/v carrier ampholytes (Fluka® brand, pH 3.0–10.0), 30 mM ARG, 1.8 mM IDA, and 0.8% v/v of p*I* synthetic peptide markers. Store the master mix at 4 °C.

3 Methods

All solutions should be prepared using ultrapure water. Solutions should also be filtered using a 0.22 μm syringe filter unit to eliminate any particulate present in solution. Reagents should be stored at room temperature unless otherwise indicated. Disposal of waste should follow regulatory procedures specified by the respective governing institution.

3.1 Capillary Gel Electrophoresis Method 1 [16]

It should be noted that several commercial kits are available for performing CGE reliably. The two methods presented here involve two styles of non-cross-linked gel (*see* **Note 3**). The mass ranges over which gels separate proteins depend on gel percentage (*see* **Note 4**).

1. Cut a capillary (I.D. 100 μm) to a length of 57 cm (*see* **Note 1**).
2. Burn in a window using a window burning tool or using two disposable glass pipets (*see* **Note 5**).
3. Thread the capillary into the cartridge.
4. Set up a CE method as shown below:

Event	Value	Duration	Solution
Rinse–pressure	25.0 psi	5.00 min	Background electrolyte solution
Inject–pressure	1.0 psi	4.0 s	Sample solution
Separate–voltage	20.0 kV	60.00 min	Background electrolyte solution
Autozero	–	–	–

5. Rinse the capillary with 0.01 M NaOH for 5 min at 25 psi, followed by 3 min with water at 25 psi before each run. Rinses are an important step to be performed before/between each run (*see* **Note 6**).
6. Use a UV lamp for CE, set to a wavelength of 254 nm (*see* Fig. 1).

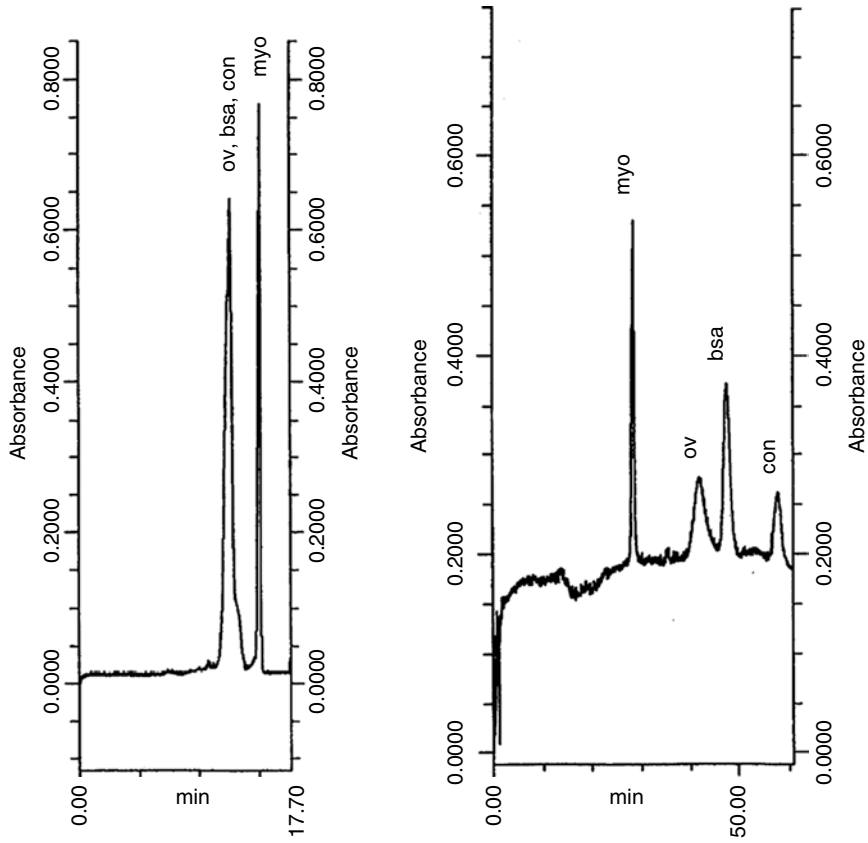

Fig. 1 (*Left*) SDS-protein complexes electropherogram where capillary does not contain polyacrylamide gel. The capillary is 57 cm long, with an id of 100 μm. Electrophoresis was run in 0.05 M phosphate buffer at pH 7, containing 0.5 % SDS, at 20 kV. *ov* ovalbumin, *bsa* bovine serum albumin, *con* conalbumin, *myo* myoglobin (*right*). SDS-protein complexes electropherogram where capillary is filled with non-cross-linked linear polyacrylamide (10 %). Electrophoresis was run in 0.05 M phosphate buffer at pH 5.5, containing 0.5 % SDS. Reprinted from Journal of Chromatography, 549, Widhalm, A., Schwer, C., Blaas, D., Kenndler, E., Capillary zone electrophoresis with a linear, non-cross-linked polyacrylamide gel: separation of proteins according to molecular mass, 446–451, Copyright (1991), with permission from Elsevier

3.2 Capillary Gel Electrophoresis Method 2 [17]

1. Cut a capillary (I.D. 75 μm) to a length of 45 cm (*see* **Note 1**).
2. Burn in a window (*see* **Note 5**).
3. Thread the capillary into the cartridge.
4. Rinse the capillary using 0.01 M NaOH for 30 min at 30 psi, and then using deionized water for 15 min at 30 psi.
5. Introduce the gel solution into the capillary using pressure at 25 psi for 5 min. Allow the capillary to equilibrate for 1 h to allow the gel to set.

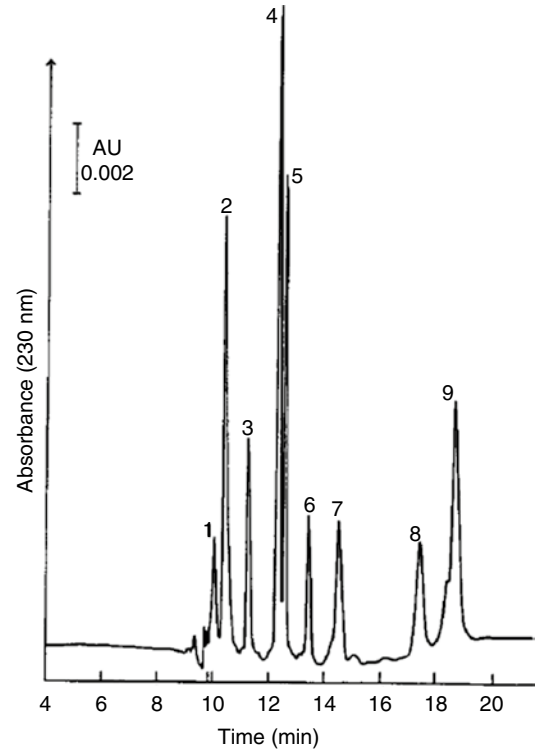

Fig. 2 Electropherogram of separated protein mixture in capillary filled with 6 % polyacrylamide. Total capillary length is 45 cm, with a length to the detector of 25 cm, and capillary id is 75 μm. Separation voltage is 12 kV, and running buffer is 0.1 M Tris–HCl and 0.25 M borate (pH 8.1). Peak: (1) aprotinin (from bovine heart), (2) cytochrome c (from horse heart), (3) trypsin inhibitor (from soybean), (4) trypsinogen (treated with phenylmethylsulfonyl fluoride (PMSF)), (5) carbonic anhydrase, (6) glyceraldehyde-3-phosphatedehydrogenase, (7) albumin (from chicken egg), (8) albumin (bovine), (9) conalbumin. Reprinted from Journal of Chromatography, 608, Wu, D. and Regnier, F. E., Sodium dodecyl sulfate-capillary gel electrophoresis of proteins using non-cross-linked polyacrylamide, 349–356, Copyright (1992), with permission from Elsevier

6. Set up a CE method as shown below:

Event	Value (kV)	Duration	Solution
Separate–voltage[a]	5.0	5.00 min	Tris-borate buffer
Inject–voltage	5.0	10.0 s	Sample solution
Separate–voltage	12.0	25.00 min	Tris-borate buffer
Autozero	–	–	–

[a] *See* **Note** 7

7. Use a PDA lamp for CE, set to wavelengths of 230 and 280 nm (*see* Fig. 2).

3.3 Capillary Isoelectric Focusing (cIEF) [13]

1. For Protein Analysis in cIEF, neutral capillaries should be used, since protein-wall interactions with bare fused silica (BFS) can affect the resolution and cause peak tailing. Use a 50 mm I.D., 50 cm total length, neutral coated capillary (Beckman Coulter) with premade UV window at 40 cm from the inlet (*see* **Note 8**). Alternatively, a neutral coating procedure can be carried out on regular BFS capillary (*see* **Note 9**).
2. Thread the capillary into the cartridge.

   Given the complexity that is often present with protein samples, especially a proteome and/or a peptidome including the presence of high-salt content in the biological systems, sample pretreatment is preferred to minimize matrix effects and obtain reproducible results [18] (*see* **Note 10**). Buffer replacement for mAb or other protein samples with membrane centrifugation is often needed. This replaces the formulation buffer components of the protein sample prior to cIEF separation.
3. Transfer 500 μL of protein sample (~5–10 mg/mL) into a Microcon-10 kDa Centrifugal Filter Unit with Ultracel-10 membrane and centrifuge at 12,000 G-force for 10 min using a benchtop centrifuge.
4. Remove the filtrate and add 250 μL of buffer replacement solution to the top of the filter. Centrifuge at 12,000 G-force for 10 min. Repeat three times.
5. Dissolve the retentate in 500 μL of buffer replacement solution by inverting the Microcon filter in a collection tube and centrifuge for 3 min at 3000 G-force. For storage, aliquot into 10 μL (~50 μg) fractions and store at −20 °C.
6. At time of analysis, thaw a protein sample aliquot and dilute with 236 μL of cIEF master mix, vortex mix, load into PCR vial.

   If buffer replacement is not needed, then in lieu of **steps 4–6**, a solution of 2 mL 1% w/v myoglobin can be prepared in water and mixed with 48 mL of master mix for analysis of standard samples.
7. Condition the neutral capillary at the start of each day with the following successive rinses. This is a general rinsing procedure for use prior to any cIEF run with a neutral capillary. Set up CE method as shown below:

Event	Value (psi)	Duration (min)	Solution
Rinse–pressure	50	5.00	Deionized water
Rinse–pressure	50	2.00	350 mM acetic acid (same as chemical mobilizer solution)
Rinse–pressure	50	5.00	cIEF polymer solution

8. Before loading a recombinant therapeutic protein sample, complete the following additional CE rinsing procedure:

Event	Value (psi)	Duration (min)	Solution
Rinse–pressure	50	3.00	Capillary cleaning solution
Rinse–pressure	50	2.00	Deionized water

9. Load the sample solution/cIEF master mix into the capillary for 99.9 s at 25 psi, allowing excess to go into a waste container in the outlet tray.

Event	Value	Duration (s)	Solution
Inject–pressure	25	99.9	Sample solution and cIEF mix

10. Once the sample and cIEF master mix have been injected, immerse the ends of the capillary into the anolyte (outlet) and catholyte (inlet).
11. Apply a separation voltage of 25 kV to the inlet of the capillary.
12. Focus for 15–25 min. When the current drops to its minimum, the proteins are focused. The focusing time will differ depending on length of capillary and viscosity of solution, as well as concentration and nature of analytes (*see* **Note 10**).
13. Replace the catholyte solution at the inlet with the chemical mobilizer solution (350 mM acetic acid). This is a method of chemical mobilization. An alternative method would be to use pressure to mobilize the sample through the detector window. A PDA or UV detector can be used with the absorbance filter at 280 nm.
14. Apply a potential of 30 kV for 30 min and collect the electropherogram (*see* Fig. 3).
15. Run a shutdown method at the end of each day to preserve the capillary and the instrument.

Event	Value (psi)	Duration (min)	Solution
Rinse–pressure	50	2.00	Deionized water
Rinse–pressure	50	10.00	CIEF polymer solution
Lamp–off			

16. Store the capillary with both ends immersed in deionized water.

    In order to minimize contamination, it is good practice in cIEF to replace the electrolyte, mobilizer, and sample solutions every five runs.

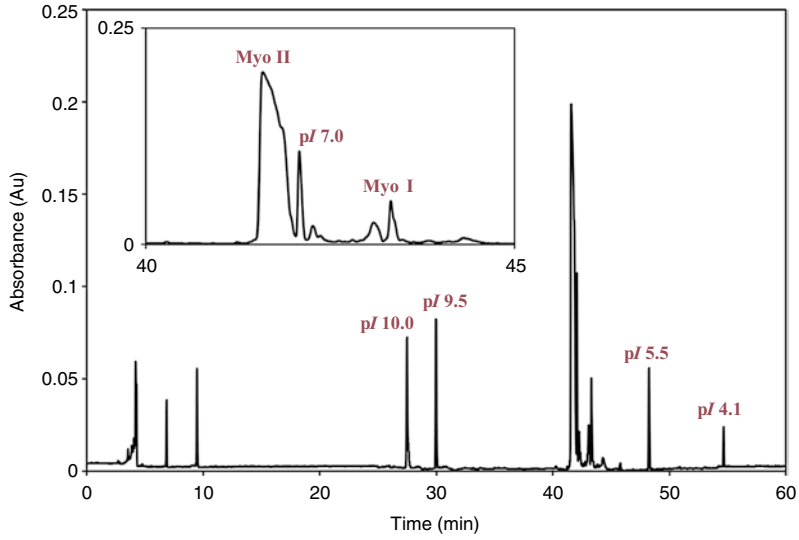

Fig. 3 A representative electropherogram of a complete cIEF experiment using p*I* 3–10 carrier ampholytes and 5 peptide p*I* standards at p*I* 10.0, 9.5, 7.0, 5.5, and 4.1. Two myoglobin isoforms were observed as labeled in the inset. Inset: magnified electropherogram from 40 to 45 min

4 Notes

1. When cutting the appropriate length of capillary it is important to ensure that the capillary ends are cut flat without jagged ends.
2. For the commonly used carrier ampholyte mixture with $3 < pI < 10$ the compounds iminodiacetic acid (IDA) and L-arginine (ARG) are appropriate sacrificial ampholytes. There are many other suitable options, and for other pH carrier ampholyte mixtures see the isoelectric buffers listed in Lalwani and Vigh's work [19].
3. Non-cross-linked gels are simply polyacrylamide, long strands of acrylamide monomers. Cross-linked acrylamide uses a crosslinker to form links between strands of polyacrylamide. In order to form a stable cross-linked gel within a capillary, it must be chemically attached to the inner wall of the capillary to prevent the gel from being extruded from the capillary [10]. Attaching the gel to the capillary wall can be a time-consuming and/or challenging task. Cross-linked gels are also difficult to form reliably in a capillary, due to their tendency to form bubbles as polymerization occurs and the gel shrinks [10, 16]. Therefore, only non-cross-linked gel methods were presented here.

4. In order to separate proteins over different mass ranges, gels of various acrylamide percentages may be used. Typically, higher percentage gels are better for lower molecular weight proteins, and lower percentage gels are better for higher molecular weight proteins [20].
5. Window burning tools for the removal of the capillary polyimide coating are commercially available, and feature a small coil element set to specific lengths to burn off the coating. However, this can also be accomplished without a machine. To remove the polyimide coating from a length of capillary, begin by attaching two disposable glass Pasteur pipets to two level and moveable surfaces, such as small boxes, with tape. Then place the two tapered ends of the pipets facing each other at a suitable distance. Thread the capillary through the wide end of one pipet into the narrow end of the other, so that the capillary is spanned across the two pipets, and a small amount of the capillary is between the two spaced pipet tips. Adjust the placement of the capillary such that the small length of capillary that is exposed between the two pipets is approximately where the window should be located. Use a lighter to burn away the capillary coating at that location for several seconds. Once the capillary coating has blackened and a very small amount of the coating has been removed, take the capillary out of the pipets and use a tissue soaked with methanol to gently wipe away the burned polyimide coating from the window. Remember to wear gloves when handling methanol.
6. Conditioning the capillary and rinsing between runs are both important steps. Conditioning ensures that the inner surface is uniformly charged. Rinses between runs ensure that any remaining analytes have been removed prior to injecting a new sample, and ensure that the inner wall of the capillary is regenerated. It can remove any gradients that may have formed. Rinsing a previously used capillary before runs will ensure that the surface becomes well wetted again. Conditioning or rinsing the capillary helps to ensure reproducibility between runs and days for both migration time and peak area by maintaining a reproducible electro-osmotic flow (EOF).
7. Separate–Voltage is used in place of Rinse–Pressure in this method in order to avoid using pressure to push the formed gel out of the capillary.
8. Neutral coated capillaries are available from Beckman.com, part number (PN) 477441 [13]
9. Neutral capillary coatings–HPC Coating Procedure. This method was adapted from the method published by Shen and Smith in 1999 [21]. Set up the following CE method:

Event	Value (psi)	Duration (min)	Solution
Rinse–pressure	20	5.00	Methanol
Rinse–pressure	20	5.00	Deionized water
Rinse–pressure	20	20.00	0.1 M NaOH
Rinse–pressure	20	5.00	Deionized water
Rinse–pressure	20	20.00	0.1 M HCl
Rinse–pressure	20	5.00	Deionized water
Rinse–pressure	20	5.00	Methanol
Rinse–pressure	30	10.00	Air
Separate–pressure[a]	50	20.00	10% HPC solution
Separate–pressure[a]	50	60.00	Air

[a]These steps are set as "separate" rather than "rinse" due to some CE instruments requiring a separation event for a method to run.

For coating the HPC polymers on the inner capillary wall the column had 25–30 psi of Nitrogen gas going through it in a GC oven and the oven was then heated at 60 °C for 20 min, and then increase the temperature from 60 to 140 °C at 5 °C per minute, and hold the temperature at 140 °C for 2 h. Allow the temperature to go back down to 60 °C on the gas chromatography (GC) oven. At this point the oven can be turned off, while the capillary is still in the oven and connected. Immerse one end in a vial with deionized water in it and there should be bubbling, which indicates that it is good to go ahead with the capillary. Once the capillary is removed from the GC oven, put it back in the CE instrument and flush it with water at 20 psi for 20.00 min. Store the capillary with both ends immersed in water.

10. During a cIEF process, when the current drops to its minimum the proteins are focused into bands in accordance to their p*I*. At this time they are neither positively nor negatively charged. They have also relatively high concentrations. This can cause them to precipitate, which is problematic since it could lead to partial clogging of the capillary, chemical mobilization failure as well as irreproducibility of analysis. To enhance protein solubility, urea, at various concentrations, is usually added to the sample mixture [1].

References

1. Zhao SS, Chen DD (2014) Applications of capillary electrophoresis in characterizing recombinant protein therapeutics. Electrophoresis 35(1):96–108
2. Leader B, Baca QJ, Golan DE (2008) Protein therapeutics: a summary and pharmacological classification. Nat Rev Drug Discov 7:21–39

3. Abou-Zeid C, Filley E, Steele J et al (1987) A simple new method for using antigens separated by polyacrylamide gel electrophoresis to stimulate lymphocytes in vitro after converting bands cut from Western blots into antigen-bearing particles. J Immunol Methods 98:5–10
4. Thongbooknkerd V, McLeish KR, Arthur JM et al (2002) Proteomic analysis of normal human urinary proteins isolated by acetone precipitation or ultracentrifugation. Kidney Int 62:1461–1469
5. Svec F, Fréchet JMJ (1995) Modified poly(glycidyl methacrylate—co-ethylene dimethacrylate) continuous rod columns for preparative-scale ion-exchange chromatography of proteins. J Chromatogr A 702: 89–95
6. Cuatrecasas P, Wilchek M, Anfinsen CB (1968) Selective enzyme purification by affinity chromatography. Biochemistry 61:636–643
7. Landers JP (ed) (2008) Handbook of capillary and microchip electrophoresis and associated microtechniques, 3rd edn. CRC Press, Boca Raton, USA
8. Guttman A (2004) The evolution of capillary gel electrophoresis: from proteins to DNA sequencing. LCGC N Am 22:896–904
9. Zhu Z, Lu JJ, Liu S (2012) Protein separation by capillary gel electrophoresis: a review. Anal Chim Acta 709:21–31
10. Guttman A (1996) Capillary sodium dodecyl sulfate-gel electrophoresis of proteins. Electrophoresis 17:1333–1341
11. Mikšik I, Sedláková P, Mikuliková K et al (2006) Matrices for capillary gel electrophoresis—a brief overview of uncommon gels. Biomed Chromatogr 20:458–465
12. North RY, Vigh G (2008) Determination of the operational pH value of a buffering membrane by an isoelectric trapping separation of a carrier ampholyte mixture. Electrophoresis 29:1077–1081
13. Mack S, Cruzado-Park I, Chapman J et al (2009) A systematic study in CIEF: defining and optimizing experimental parameters critical to method reproducibility and robustness. Electrophoresis 30:4049–4058
14. Wu X, Pawliszyn J (2002) Whole-column imaging capillary electrophoresis of proteins with a short capillary. Electrophoresis 23:542–549
15. Zhong X, Maxwell EJ, Ratnayake C et al (2011) Flow-through microvial facilitating interface of capillary isoelectric focusing and electrospray ionization mass spectrometry. Anal Chem 83:8748–8755
16. Widhalm A, Schwer C, Blaas D et al (1991) Capillary zone electrophoresis with a linear, non-cross-linked polyacrylamide gel: separation of proteins according to molecular mass. J Chromatogr 549:446–451
17. Wu D, Regnier FE (1992) Sodium dodecyl sulfate—capillary gel electrophoresis of proteins using non-cross-linked polyacrylamide. J Chromatogr 608:349–356
18. Zhao SS, Zhong X, Tie C, Chen DDY (2012) Capillary electrophoresis-mass spectrometry for analysis of complex samples. Proteomics 12:2991–3012
19. Lalwani S, Vigh G (2005) Isoelectric buffers, part 3: determination of pKa and pI values of diamino sulfate carrier ampholytes by indirect UV-detection capillary electrophoresis. Electrophoresis 26:2503–2510
20. Thermo Scientific. General recommendations for SDS-PAGE: protocol. http://www.thermoscientificbio.com/uploadedFiles/Resources/general-recommendations-for-sds-page.pdf. Accessed 25 Aug 2014
21. Shen Y, Smith RD (2000) High-resolution capillary isoelectric focusing of proteins using highly hydrophilic-substituted cellulose-coated capillaries. J Microcolumn Sep 12(3):135–141

Chapter 12

Characterization of Chemical and Physical Modifications of Human Serum Albumin by Capillary Zone Electrophoresis

Anne-Lise Marie, Nguyet Thuy Tran, and Myriam Taverna

Abstract

Therapeutic proteins can easily undergo chemical or physical changes during their manufacturing, purification, and storage. These modifications might change or reduce their biological activity. Therefore, it is important to have analytical methodologies that are able to reliably detect, characterize, and quantify degradation products in formulations. Capillary Zone Electrophoresis (CZE) is very well suited for the analysis of proteins due to its relatively easiness of implementation, separation efficiency, and resolving power. We describe here a CZE method that allows separating more than nine forms in therapeutic albumin, including oxidized, glycated, and truncated forms. This method uses a polyethylene oxide (PEO) coating and a buffer composed of HEPES and SDS at physiological pH. The method is reproducible (RSD < 0.5 and 4% for migration times and peak areas, respectively) and allows quantitation of albumin forms in pharmaceutical preparations.

Key words Human serum albumin, Oxidized albumin, Glycated albumin, Truncated forms, Capillary zone electrophoresis, Polyethylene oxide coating

1 Introduction

Human serum albumin (HSA) is a therapeutic protein used to correct hypovolemia and hypoalbuminemia, which are symptoms associated with liver failure, renal diseases, or sepsis. Apart from maintaining oncotic pressure, HSA exhibits many other important physiological functions such as transport of endogen and exogen ligands (fatty acids, hormones, bilirubin, drugs…), and powerful antioxidant activity mostly due to its single free cysteine (Cys34) [1]. The commercial HSA preparations, obtained from fractionation of human plasma, are very heterogeneous. Indeed, during biotechnological production and storage, the HSA molecule can undergo various physical or chemical modifications like aggregation, cleavage, and oxidation [2]. The donor population represents another source of heterogeneity. For example, it has been shown that albumin undergoes increased glycation in diabetic subjects [3],

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and liver diseases are accompanied by high levels of cysteinylated or sulfonic HSA forms [4]. These modifications may affect the binding properties of the molecule, and thereby have an impact on its therapeutic activity. Analytical methods are therefore required to detect, characterize, and quantify the different forms present in HSA preparations. Recently, our laboratory has developed a capillary zone electrophoresis (CZE) method, which allows to separate and quantify more than nine HSA forms in a commercial preparation [2]. After a brief overview summarizing the different chemical or physical modifications that a therapeutic protein may undergo, this chapter will focus on HSA, with a detailed description of the developed CZE protocol.

1.1 Chemical and Physical Modifications in Therapeutic Proteins

The clinical use of therapeutic proteins has enabled the treatment of a wide range of life-threatening diseases. Many of these diseases were considered incurable only a few decades ago. Almost half of the new drugs recently approved by the United States Food and Drug Administration (FDA) are therapeutic proteins [5]. Due to their unique physico-chemical properties, therapeutic proteins are prone to modifications such as denaturation, aggregation, oxidation, deamidation, racemization, fragmentation, or glycation. These modifications may lead to a potential loss of therapeutic efficacy or unwanted immunogenic reactions.

1.1.1 Physical Modifications

Proteins, because of their polymeric nature and their ability to form superstructures (e.g., secondary, tertiary, and quaternary), can undergo structural changes without any change in their chemical composition [6, 7]. These modifications include denaturation, aggregation, and precipitation.

Denaturation corresponds to the loss of the globular or three-dimensional structure of the protein. This globular structure is referred to as the native state, which, in most cases, is the active form of the protein [6]. The denaturation phenomenon induces the unfolding of the protein, which may lead to its aggregation, and even to its precipitation. A variety of factors like heat or light exposure, lyophilization, freezing/thawing, organic solvents, salts, and chaotropic agents may cause denaturation [8, 9]. Elevated temperature is probably the most common stress that causes the unfolding of the protein. Heat-induced denaturation is often irreversible as the unfolded proteins rapidly associate to form aggregates [6, 10]. High concentration of chaotropes (e.g., urea) is another important cause of denaturation. Chaotropic agents denature proteins into a totally unordered and unfolded state [9, 11–13]. Recently, much more attention has been paid to less common phenomena like cold denaturation [6, 14] and pressure-induced denaturation [6, 15, 16]. The main consequence of protein denaturation is a loss of therapeutic activity, but cytotoxic effects have also been observed with several biotherapeutic proteins [5, 10].

Proteins may aggregate through several mechanisms, classified as soluble/insoluble, covalent/noncovalent, reversible/irreversible, and native/denatured. For therapeutic proteins, the presence of aggregates is undesirable because the aggregates may lead to a loss of activity, an immunogenic reaction, or cause adverse effects during administration [17, 18]. *Aggregation* is a phenomenon routinely encountered during purification, sterilization, formulation, and storage. Stresses to the proteins such as freezing/thawing, ultrafiltration/diafiltration, agitation, exposure to air or interactions with metal surfaces [6, 10], but also bioprocess-related impurities may result in the formation of aggregates [19, 20]. The size of protein aggregates varies from soluble submicron range to visible precipitates. Numerous proteins have strong tendency to aggregate. For example, insulin is prone to aggregate under various conditions [21, 22]. We can also mention the serpins, which typically aggregate when polymerized in vitro under heat exposure or low concentrations of chaotropes [23, 24].

1.1.2 Chemical Modifications

Chemical modifications correspond to the change or loss of specific functional groups or the breakage of covalent bonds. They encompass oxidation, deamidation, racemization, fragmentation, and glycation.

Protein *oxidation* is a covalent modification of amino acids that is induced by reactive oxygen species [6]. This chemical degradation is catalyzed by metals and light. Almost all amino acids can be oxidized, but cysteine and methionine, the two sulfur-containing amino acids, are particularly prone to oxidation. Cysteine oxidation may lead to the formation of disulfide linkages [25], but also to a wide variety of oxidized forms (*see* Subheading 1.2). Methionine can be oxidized into sulfoxides, and, in more drastic conditions, sulfone forms. The list of oxidants that directly or indirectly attack methionine is large and includes chloramines, hydrogen peroxide, hypochlorous acid, oxygen, and ozone [26]. Methionine oxidation is observed in many therapeutic proteins. For example, the recombinant human growth factor filgrastim contains four methionine residues susceptible to be oxidized to their sulfoxide derivatives, three of which are more prone to oxidation because they can interact with solvent molecules, whereas the fourth methionine is located at a hydrophobic region of the protein [27]. Nowadays, it is well recognized that oxidation of therapeutic proteins may limit their clinical efficiency or stability.

Deamidation is a spontaneous nonenzymatic process, which involves either asparagine (Asn) or glutamine (Gln) residues. During deamidation, an Asn residue is converted into aspartic (Asp) or isoaspartic (isoAsp) acid, whereas a Gln residue is converted into glutamic acid. The reaction produces an intermediate compound which is a cyclic succinimide. The rates of deamidation depend on the primary sequence and 3D structure of the protein, and on solution properties like pH, temperature, or ionic strength [28].

Deamidation has been observed and characterized in a wide variety of proteins, such as recombinant growth factors or hormones [29, 30] and IgG antibodies [31, 32].

Racemization is the transformation of *L*-Asp (or *L*-isoAsp) into *D*-Asp (or *D*-isoAsp). It is a chemical modification which is related to deamidation, since it follows the same chemical pathway with the formation of a succinimide intermediate. Racemization may be induced by high temperature or pH stresses [8]. For example, racemization of aspartate residue in a thermally stressed antibody has been reported [33].

Fragmentation corresponds to disruption of a covalent bond in a protein. This degradation is a function of the primary sequence, the flexibility of the local structure, the solvent conditions (pH, temperature), and the presence of metals or radicals [34]. Two distinct mechanisms may induce fragmentation: hydrolysis and β-elimination. For example, the hinge region of monoclonal antibodies may undergo fragmentation by direct hydrolysis [35] or β-elimination [36].

Glycation is a slow nonenzymatic reaction that initially involves the addition of a reducing sugar, or its derivatives, to amine groups of proteins. This leads to the formation of a Schiff base, which can subsequently undergo rearrangement into irreversible conjugates, called advanced glycation end products (AGEs) [6, 37]. The formation of AGEs affects the structure and functionality of proteins. It has been reported that excipient like sucrose could potentially cause protein glycation, due to the formation of glucose and fructose upon sucrose hydrolysis [38–40].

Although it is very convenient to distinguish chemical from physical modifications, the fact remains that they may be interrelated [6]. For example, methionine oxidation can lead to destabilization of the native structure of a given protein, and thereby increase its aggregation rate. This has been reported for recombinant human growth hormones [41] and immunoglobulines [42].

1.2 Chemical and Physical Modifications in HSA Preparations

The commercial HSA preparations, obtained from fractionation of human plasma, are very heterogeneous. Indeed, for intrinsic (natural source) or extrinsic reasons (bioprocess, storage), the HSA molecule can undergo various physical or chemical modifications like aggregation, polymerization, truncation, oxidation, and glycation.

It has been reported that the bioprocessing steps may affect the stability of the HSA molecules obtained from pooled human plasma. In particular, thermal treatments like viral inactivation and lyophilization perturb the secondary structure of HSA, leading to its *aggregation* [43–45].

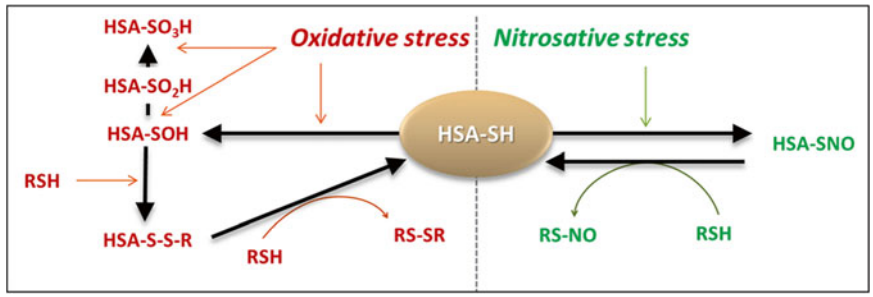

Fig. 1 Overview of the steps leading to Cys34 oxidation and thiolation (highlighted in *red*). Steps involved in the nitrosylation of Cys34 of HSA are highlighted in *green*. Formation of higher oxidation states of HSA is also shown (issued from [1], with permission)

HSA dimerization and *polymerization* are likely to occur during purification and storage, and are often correlated with the aggregation process [46–48]. It has been shown that dimer and polymer formation may contribute to rare instances of allergic reactions [49].

The HSA molecule can be cleaved by reaction with proteases. The first *truncated forms* described in the literature were HSA-L (loss of leucine from the C-terminus) and HSA-DA (loss of aspartic acid and alanine from the N-terminus) [46, 50, 51], but recently many other truncated forms have been reported by our group [2].

The free sulfhydryl residue (SH) of the Cys34 of native albumin is implicated in a great number of *oxidation* reactions [52]. For instance it can bind other thiol-containing compounds like cysteine, homocysteine, or glutathione (leading to the so-called HSA + Cys, HSA + HCys, and HSA + G species, respectively) [50, 53, 54], interact with nitric oxide (NO) and thereby form S-nitrosothiols (HSA-SNO) [55], or be oxidized into sulfenic (HSA-SOH), sulfinic (HSA-SO_2H), or sulfonic (HSA-SO_3H) acids [1, 56, 57] (Fig. 1).

In addition, serum albumin is a plasma protein highly sensitive to *glycation* in the blood. Numerous studies have identified the main sites on HSA molecule modified by glycation in vivo [58, 59]. Because of their high nucleophile properties, lysine, arginine, and cysteine are the sole residues prone to glycation. Figure 2 describes the different structures of AGEs reported in the literature [60, 61]. Recently, our group has characterized, in commercial albumin, new AGE derivatives that had not been reported yet [2].

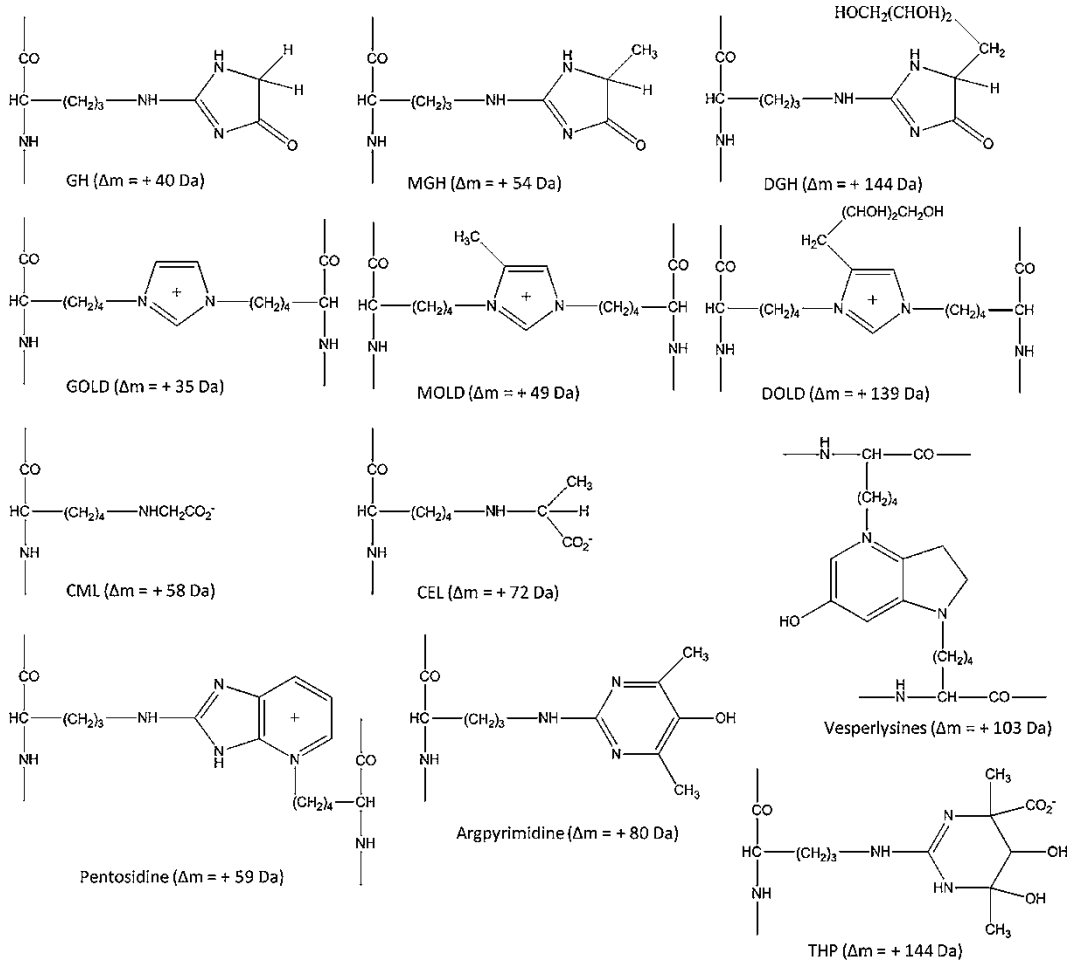

Fig. 2 Examples of AGE chemical structures of proteins (Δm, mass difference between AGE derivative and native protein). AGEs derived from lysine residues: GOLD, MOLD, DOLD, CML, CEL, and Vesperlysines. AGEs derived from arginine residues: GH, MGH, DGH, Argpyrimidine, Pentosidine, and THP (issued from [2], with permission)

2 Materials

All buffers and samples are prepared with Milli-Q water using a Direct-Q 3 UV purification system (Millipore) and are filtered through a 0.2 μm Millex membrane (Millipore) before use.

2.1 Instrument and Capillary

- CE instrument: P/ACE™ 5500 (Beckman Coulter) equipped with UV detector.
- During the CE analysis, the UV detection is performed at 214 nm.
- The internal and external diameters of the capillary are 50 and 375 μm, respectively.
- The total and effective lengths of the capillary are 57 and 50 cm, respectively.

2.2 Preparation of PEO Solution at 2.22 mg/mL for Capillary Coating

- Add 20 mL of water in a 50 mL volumetric flask.
- Weigh 111 mg of polyethylene oxide (PEO, MW 200,000 g/mol) and transfer it into the volumetric flask.
- Adjust the volume to 50 mL with water, and mix the solution with a stirrer for 2 h (*see* **Note 1**).
- Every day prepare a fresh solution containing 0.2 % PEO (w/v) in 0.1 N HCl by mixing 4.5 mL of 2.22 mg/mL PEO solution with 0.5 mL of 1 N HCl.

2.3 Preparation of Buffers for CE Analyses

2.3.1 Buffer Used for EOF Measurement: 20 mM Sodium Phosphate pH 7.4

- Add a few mL of water in a 50 mL volumetric flask.
- Weigh 120 mg of sodium dihydrogen phosphate (NaH_2PO_4, MW 120 g/mol) and transfer it into the volumetric flask.
- Mix and adjust pH at 7.4 with 1 N NaOH.
- Adjust the volume to 50 mL with water.

2.3.2 Buffer Used for HSA Analyses: 50 mM HEPES, 0.5 mM SDS, pH 7.5

10 mM SDS Solution

- Add a few mL of water in a 50 mL volumetric flask.
- Weigh 144 mg of Sodium Dodecyl Sulfate (SDS, MW 288.38 g/mol) and transfer it into the volumetric flask.
- Add water to a volume of 50 mL.

50 mM HEPES Solution with 0.5 mM SDS, pH 7.5

- Add 20 mL of water in a 50 mL volumetric flask.
- Weigh 595 mg of 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES, MW 238.30 g/mol) and transfer it into the volumetric flask.
- Add 1170 µL of 1 N NaOH (*see* **Note 2**).
- Add 2.5 mL of the 10 mM SDS solution.
- Mix and adjust pH at 7.5 with 1 N NaOH.
- Adjust the volume to 50 mL with water.

2.4 Sample Preparations

2.4.1 Neutral Marker Solution: 1 g/L of Thiourea (*See* Note 3)

- Add a few mL of water in a 10 mL volumetric flask.
- Weigh 10 mg of thiourea (MW 76.12 g/mol) and transfer it into the volumetric flask.
- Adjust to 10 mL with water, and mix the solution until complete solubilization using a stirrer.

2.4.2 HSA Solutions

- The commercial albumins were provided by LFB Biotechnologies (Les Ulis, France) or by other companies. Albumin concentration in these preparations was 200 or 250 mg/mL (*see* **Note 4**).
- The commercial albumins were stored at −20 °C before use, and were placed in fridge at 8 °C after thawing (*see* **Note 5**).

0.05 M Solution of Copper Nitrate

- Add a few mL of water in a 10 mL volumetric flask.
- Weigh 93.8 mg of copper nitrate ($Cu(NO_3)_2$, MW 187.56 g/mol) and transfer it into the volumetric flask.
- Adjust to 10 mL with water, and mix by hand until complete solubilization of the copper ions (the obtained solution is slightly colored in blue).

1 mg/mL HSA with 30 μM of Copper Nitrate Freshly Prepared Each Day (*See* Note 6)

For the albumin preparations at 200 mg/mL, add 25 μL of the albumin solution to 4972 μL of water (the quantity of albumin prepared before injection depends on the available quantity of commercial albumin, by consequence this volume may be modified). Then, add 3 μL of the 0.05 M $Cu(NO_3)_2$ solution (for albumin preparations at 250 mg/mL, proceed in the same way by adapting the volumes).

3 CZE Method

3.1 Coating Procedure

Each new bare fused silica capillary is systematically treated with the following sequence: water (5 min), 1 N NaOH (5 min), 0.1 N NaOH (5 min), and water (5 min), by applying 20 psi for each rinse.

- Apply the coating procedure: water (5 min), 0.1 N NaOH (5 min), 1 N HCl (10 min), water (10 min), 0.2% PEO solution (5 min), and water (5 min), by applying 20 psi for each rinse.

3.2 Inter-day PEO Regeneration

Each day, before performing the analyses, make a regeneration of the PEO coating by applying the following sequence of rinses (20 psi each): water (3 min), 1 N HCl (5 min), 0.2% PEO solution (5 min), electrolyte buffer (3 min) (*see* **Note 7**).

3.3 EOF Measurement Before Coating

- Rinse the capillary with 20 mM sodium phosphate pH 7.4 (*see* **Note 8**) (3 min at 20 psi).
- Inject a plug of thiourea at 1 g/L (3 s at 0.3 psi) from the inlet of the capillary.
- Apply a voltage of +25 kV for 10 min.
- Determine the EOF mobility according to the equation: $\mu_{EOF} = (L_{eff} L_{tot}) / (V t_m)$, where μ_{EOF} corresponds to electroosmotic flow mobility, L_{eff} and L_{tot} are effective and total lengths, respectively, V is the applied voltage, and t_m is the migration time of the analyte.

3.4 EOF Measurement After Coating

- Rinse the capillary with 20 mM sodium phosphate pH 7.4 (*see* **Note 9**) (3 min at 20 psi).
- Inject a plug of thiourea at 1 g/L (3 s at 0.3 psi) from the outlet of the capillary (*see* **Note 10**).

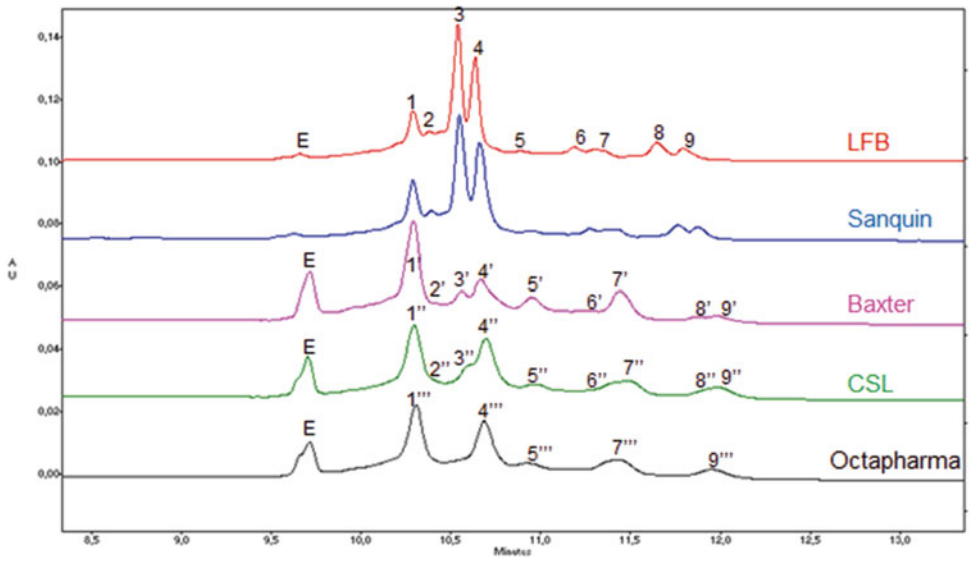

Fig. 3 Electropherograms of five competitive HSA preparations. Analytical conditions: PEO coated capillary, 57 cm (50 cm to detector) × 50 μm I.D. BGE: 50 mM HEPES, 0.5 mM SDS, pH 7.5. The peak noted *E* corresponds to excipient contained in the therapeutic albumin (issued from [2], with permission)

- Apply a negative voltage of −25 kV for 70 min.
- Determine the EOF mobility with the equation described above (*see* **Note 11**).

3.5 HSA Analysis

- Before each HSA injection, make an inter-run PEO regeneration, as followed: water (3 min), 1 N HCl (3 min), 0.2% PEO solution (5 min), and electrolyte buffer (3 min).
- Inject the commercial albumin sample for 5 s at 0.5 psi from the inlet of the capillary.
- Apply a negative voltage of −25 kV for 30 min (*see* **Notes 12** and **13**).
- Determine the migration times and areas of the different peaks separated by CZE with the instrument software (a successful analysis allows the separation of nine peaks, *see* Fig. 3) (*see* **Note 14**).

4 Notes

1. As the PEO powder may be difficult to solubilize, it is recommended to slightly heat the solution with a hot plate.
2. The addition of this volume allows to obtain a pH close to 7.5, but the pH value has to be checked and adjusted if necessary.

3. Other neutral markers can be used like DMSO or benzyl alcohol.
4. These albumin preparations contain excipients such as sodium caprylate and *N*-acetyl-L-tryptophanate in different quantity.
5. After thawing, the albumin preparations do not have to be frozen again because multiple freezing/thawing cycles can degrade the protein.
6. The commercial albumin preparation is analyzed by CE without desalting. A desalting step, to remove the excipients contained in the albumin preparation, can be undertaken but is not advised because the desalting process may involve a degradation of the protein.
7. After this PEO regeneration, HSA sample can be directly injected.
8. Another buffer can be used to determine the EOF, provided that the pH is in the range 3–8 and it does not contain high quantity of surfactants, which can alter or degrade the PEO coating.
9. Use the same buffer for EOF measurement before and after coating.
10. In this case, the effective length will be 7 cm.
11. The EOF mobility has to be reduced by a factor superior to 20. If this factor is inferior to 10, repeat the coating procedure.
12. The HSA molecule is globally negatively charged at pH 7.5, and will migrate to the anode end.
13. For an effective length of 50 cm, the HSA peaks appear around 10–15 min.
14. Repeat at least three successive analyses for each HSA sample (RSD for migration times and peak areas should be less than to 0.5 and 4 %, respectively).

Acknowledgments

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References

1. Taverna M, Marie AL, Mira JP, Guidet B (2013) Specific antioxidant properties of human serum albumin. Ann Intensive Care 3:1–7
2. Marie AL, Przybylski C, Gonnet F, Daniel R, Urbain R, Chevreux G, Jorieux S, Taverna M (2013) Capillary zone electrophoresis and capillary electrophoresis-mass spectrometry for analyzing qualitative and quantitative variations in therapeutic albumin. Anal Chim Acta 800:103–110
3. Guerin-Dubourg A, Catan A, Bourdon E, Rondeau P (2012) Structural modifications of human albumin in diabetes. Diabetes Metab 38:171–178

4. Oettl K, Marsche G (2010) Redox state of human serum albumin in terms of cysteine-34 in health and disease. Methods Enzymol 474:181–195
5. Fekete S, Beck A, Veuthey JL, Guillarme D (2014) Theory and practice of size exclusion chromatography for the analysis of protein aggregates. J Pharm Biomed Anal 101:161–173
6. Manning MC, Chou DK, Murphy BM, Payne RW, Katayama DS (2010) Stability of protein pharmaceuticals: an update. Pharm Res 27:544–575
7. Staub A, Guillarme D, Schappler J, Veuthey JL, Rudaz S (2011) Intact protein analysis in the biopharmaceutical field. J Pharm Biomed Anal 55:810–822
8. Manning MC, Patel K, Borchardt RT (1989) Stability of protein pharmaceuticals. Pharm Res 6:903–918
9. Guerini Rocco A, Mollica L, Ricchiuto P, Baptista AM, Gianazza E, Eberini I (2008) Characterization of the protein unfolding processes induced by urea and temperature. Biophys J 94:2241–2251
10. Wang W (2005) Protein aggregation and its inhibition in biopharmaceutics. Int J Pharm 289:1–30
11. O'Brien EP, Dima RI, Brooks B, Thirumalai D (2007) Interactions between hydrophobic and ionic solutes in aqueous guanidinium chloride and urea solutions: lessons for protein denaturation mechanism. J Am Chem Soc 129:7346–7353
12. Zhang Y, Cremer PS (2006) Interactions between macromolecules and ions: the Hofmeister series. Curr Opin Chem Biol 10:658–663
13. Majumdar R, Manikwar P, Hickey JM, Samra HS, Sathish HA, Bishop SM, Middaugh CR, Volkin DB, Weis DD (2013) Effects of salts from the Hofmeister series on the conformational stability, aggregation propensity, and local flexibility of an IgG1 monoclonal antibody. Biochemistry 52:3376–3389
14. Luan B, Shan B, Baiz C, Tokmakoff A, Raleigh DP (2013) Cooperative cold denaturation: the case of the C-terminal domain of ribosomal protein L9. Biochemistry 52:2402–2409
15. Harano Y, Yoshidome T, Kinoshita M (2008) Molecular mechanism of pressure denaturation of proteins. J Chem Phys 129:1–9
16. Boonyaratanakornkit BB, Park CB, Clark DS (2002) Pressure effects on intra- and intermolecular interactions within proteins. Biochim Biophys Acta 1595:235–249
17. Cromwell MEM, Hilario E, Jacobson F (2006) Protein aggregation and bioprocessing. AAPS J 8:572–579
18. Ratanji KD, Derrick JP, Dearman RJ, Kimber I (2014) Immunogenicity of therapeutic proteins: influence of aggregation. J Immunotoxicol 11:99–109
19. Wang W, Ignatius AA, Thakkar SV (2014) Impact of residual impurities and contaminants on protein stability. J Pharm Sci 103:1315–1330
20. Kumar S, Zhou S, Singh SK (2014) Metal ion leachates and the physico-chemical stability of biotherapeutic drug products. Curr Pharm Des 20:1173–1181
21. Xu Y, Yan Y, Seeman D, Sun L, Dubin PL (2012) Multimerization and aggregation of native-state insulin: effect of zinc. Langmuir 28:579–586
22. Pryor E, Kotarek JA, Moss MA, Hestekin CN (2011) Monitoring insulin aggregation via capillary electrophoresis. Int J Mol Sci 12:9369–9388
23. Huntington JA, Sendall TJ, Yamasaki M (2009) New insight into serpin polymerization and aggregation. Prion 3:12–14
24. Yamasaki M, Li W, Johnson DJD, Huntington JA (2008) Crystal structure of a stable dimer reveals the molecular basis of serpin polymerization. Nature 455:1255–1259
25. Chalker JM, Bernardes GJL, Lin YA, Davis BG (2009) Chemical modification of proteins at cysteine: opportunities in chemistry and biology. Chem Asian J 4:630–640
26. Levine RL, Moskovitz J, Stadtman ER (2000) Oxidation of methionine in proteins: roles in antioxidant defense and cellular regulation. IUBMB Life 50:301–307
27. Holzmann J, Hausberger A, Rupprechter A, Toll H (2013) Top-down MS for rapid methionine oxidation site assignment in filgrastim. Anal Bioanal Chem 405:6667–6674
28. Taichrib A, Pelzing M, Pellegrino C, Rossi M, Neusüβ C (2011) High resolution TOF MS coupled to CE for the analysis of isotopically resolved intact proteins. J Proteom 74:958–966
29. Santana H, González Y, Targon Campana P, Noda J, Amarantes O, Itri R, Beldarraín A, Páez R (2013) Screening for stability and compatibility conditions of recombinant human epidermal growth factor for parenteral formulation: effect of pH, buffers, and excipients. Int J Pharm 452:52–62
30. Haselberg R, Brinks V, Hawe A, De Jong GJ, Somsen GW (2011) Capillary electrophoresis-mass spectrometry using noncovalently coated capillaries for the analysis of biopharmaceuticals. Anal Bioanal Chem 400:295–303
31. Vlasak J, Bussat MC, Wang S, Wagner-Rousset E, Schaefer M, Klinguer-Hamour C, Kirchmeier M, Corvaïa N, Ionescu R, Beck A (2009) Identification and characterization of

asparagine deamidation in the light chain CDR1 of a humanized IgG1 antibody. Anal Biochem 392:145–154

32. Zhang YT, Hu J, Pace AL, Wong R, Wang YJ, Kao YH (2014) Characterization of asparagine 330 deamidation in an Fc-fragment of IgG1 using cation exchange chromatography and peptide mapping. J Chromatogr B 965:65–71
33. Zhang J, Yip H, Katta V (2011) Identification of isomerization and racemization of aspartate in the Asp–Asp motifs of a therapeutic protein. Anal Biochem 410:234–243
34. Vlasak J, Ionescu R (2011) Fragmentation of monoclonal antibodies. mAbs 3:253–263
35. Cordoba AJ, Shyong BJ, Breen D, Harris RJ (2005) Non-enzymatic hinge region fragmentation of antibodies in solution. J Chromatogr B 818:115–121
36. Gaza-Bulseco G, Liu H (2008) Fragmentation of a recombinant monoclonal antibody at various pH. Pharm Res 25:1881–1890
37. Vistoli G, De Maddis D, Cipak A, Zarkovic N, Carini M, Aldini G (2013) Advanced glycoxidation and lipoxidation end products (AGEs and ALEs): an overview of their mechanisms of formation. Free Radic Res 47:3–27
38. Fischer S, Hoernschemeyer J, Mahler HC (2008) Glycation during storage and administration of monoclonal antibody formulations. Eur J Pharm Biopharm 70:42–50
39. Gadgil HS, Bondarenko PV, Pipes G, Rehder D, McAuley A, Perico N, Dillon T, Ricci M, Treuheit M (2007) The LC/MS analysis of glycation of IgG molecules in sucrose containing formulations. J Pharm Sci 96:2607–2621
40. Gadgil HS, Bondarenko PV, Treuheit MJ, Ren D (2007) Screening and sequencing of glycated proteins by neutral loss scan LC/MS/MS method. Anal Chem 79:5991–5999
41. Mulinacci F, Poirier E, Capelle MAH, Gurny R, Arvinte T (2013) Influence of methionine oxidation on the aggregation of recombinant human growth hormone. Eur J Pharm Biopharm 85:42–52
42. Liu D, Ren D, Huang H, Dankberg J, Rosenfeld R, Cocco MJ, Li L, Brems DN, Remmele RL (2008) Structure and stability changes of human IgG1 Fc as a consequence of methionine oxidation. Biochemistry 47:5088–5100
43. Wetzel R, Becker M, Behlke J, Billwitz H, Böhm S, Ebert B, Hamann H, Krumbiegel J, Lassmann G (1980) Temperature behaviour of human serum albumin. Eur J Biochem 104:469–478
44. Lin JJ, Meyer JD, Carpenter JF, Manning MC (2000) Stability of human serum albumin during bioprocessing: denaturation and aggregation during processing of albumin paste. Pharm Res 17:391–396
45. Vetri V, Librizzi F, Leone M, Militello V (2007) Thermal aggregation of bovine serum albumin at different pH: comparison with human serum albumin. Eur Biophys J 36:717–725
46. Alahmad Y, Tran NT, Le Potier I, Forest E, Jorieux S, Taverna M (2011) A new CZE method for profiling human serum albumin and its related forms to assess the quality of biopharmaceuticals. Electrophoresis 32:292–299
47. Ogasawara Y, Namai T, Togawa T, Ishii K (2006) Formation of albumin dimers induced by exposure to peroxides in human plasma: a possible biomarker for oxidative stress. Biochem Biophys Res Comm 340:353–358
48. Oliva A, Santoveña A, Llabres M, Fariña JB (1999) Stability study of human serum albumin pharmaceutical preparations. J Pharm Pharmacol 51:385–392
49. Quinlan GJ, Martin GS, Evans TW (2005) Albumin: biochemical properties and therapeutic potential. Hepatology 41:1211–1219
50. Bar-Or D, Bar-Or R, Rael LT, Gardner DK, Slone DS, Craun ML (2005) Heterogeneity and oxidation status of commercial human albumin preparations in clinical use. Crit Care Med 33:1638–1641
51. Bar-Or D, Rael LT, Bar-Or R, Slone DS, Craun ML (2006) The formation and rapid clearance of a truncated albumin species in a critically ill patient. Clin Chim Acta 365:346–349
52. Kawakami A, Kubota K, Yamada N, Tagami U, Takehana K, Sonaka I, Suzuki E, Hirayama K (2006) Identification and characterization of oxidized human serum albumin: a slight structural change impairs its ligand-binding and antioxidant functions. FEBS J 273: 3346–3357
53. Oettl K, Stauber RE (2007) Physiological and pathological changes in the redox state of human serum albumin critically influence its binding properties. Br J Pharmacol 151: 580–590
54. Naldi M, Giannone FA, Baldassarre M, Domenicali M, Caraceni P, Bernardi M, Bertucci C (2013) A fast and validated mass spectrometry method for the evaluation of human serum albumin structural modifications in the clinical field. Eur J Mass Spectrom 19:491–496
55. Ishima Y, Hiroyama S, Kragh-Hansen U, Maruyama T, Sawa T, Akaike T, Kai T, Otagiri M (2010) One-step preparation of *S*-nitrosated human serum albumin with high biological activities. Nitric Oxide 23:121–127
56. Turell L, Carballal S, Botti H, Radi R, Alvarez B (2009) Oxidation of the albumin thiol to sulfenic acid and its implications in the intravascular compartment. Braz J Med Biol Res 42:305–311

57. Turell L, Botti H, Bonilla L, Torres MJ, Schopfer F, Freeman BA, Armas L, Ricciardi A, Alvarez B, Radi R (2014) HPLC separation of human serum albumin isoforms based on their isoelectric points. J Chromatogr B Analyt Technol Biomed Life Sci 944:144–151
58. Anguizola J, Matsuda R, Barnaby OS, Hoy KS, Wa C, DeBolt E, Koke M, Hage DS (2013) Review: glycation of human serum albumin. Clin Chim Acta 425:64–76
59. Rondeau P, Bourdon E (2011) The glycation of albumin: structural and functional impacts. Biochimie 93:645–658
60. Wa C, Cerny RL, Clarke WA, Hage DS (2007) Characterization of glycation adducts on human serum albumin by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Clin Chim Acta 385:48–60
61. Ahmed N, Argirov OK, Minhas HS, Cordeiro CAA, Thornalley PJ (2002) Assay of advanced glycation end products (AGEs): surveying AGEs by chromatographic assay with derivatization by 6-aminoquinolyl-*N*-hydroxysuccinimidyl-carbamate and application to *Nε*-carboxymethyl-lysine- and *Nε*-(1-carboxyethyl)lysine-modified albumin. Biochem J 364:1–14

Chapter 13

Capillary Electrophoresis Method for the Assessment of Erythropoiesis-Stimulating Agents in Final Formulations

Michel Girard, Anita Kane, and Sylvie Boucher

Abstract

Capillary electrophoresis (CE) comprises several separation modes that can be used to characterize proteins in terms of physico-chemical properties such as isoelectric point or molecular weight, or in terms of purity/heterogeneity for the presence of charge or size variants. In glycoproteins the heterogeneity occurring as a consequence of variable amounts of terminal sialic acid residues on glycan moieties can be detected by CE. As such, a capillary zone electrophoresis (CZE) method was found suitable for the detection of isoforms of several erythropoiesis-stimulating agents (Bietlot and Girard, J Chromatogr A 759:177–184, 1997; Boucher et al., J Pharm Biomed Anal 71:207–213, 2012). In particular, the method can be used to analyze finished products containing erythropoietin-α, erythropoietin-β, or darbepoetin-α regardless of the formulation and without the need for sample pretreatment. The major excipients encountered in the various formulations included polysorbate 80, polysorbate 20, or human serum albumin. The ability of the method to resolve isoforms of the active ingredient in finished product enables the comparison of the isoform profile with that of the corresponding drug substance, allowing the assessment of the structural integrity and content of the active ingredients in finished products.

Key words Erythropoietin, Darbepoetin, Formulation, Capillary electrophoresis, Erythropoiesis-stimulating agent, Glycoprotein

1 Introduction

Proteins undergo post-translational modifications (PTMs) such as the attachment of glycan moieties at specific amino acids. This process is called glycosylation and results in the formation of glycoproteins. It is one of the most widely occurring PTM in eukaryotic organisms and it is widely acknowledged that more than 50 % of all proteins are glycosylated. The widespread occurrence of this process has been extensively studied and many roles have been shown for glycosylation in biological systems ranging from roles in transport, cell surface recognition, enzymatic processes, or the immune system. From a structural point of view, glycosylation at a specific amino acid is generally heterogeneous, leading to the formation of complex mixtures referred to as glycoforms. In addition, the

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presence of charged monosaccharides such as sialic acid residues at the terminal end of glycans confers to the glycoform a different overall charge and, as such, can lead to it being separated from its nonsialylated form using appropriate methods. Further information on the structural aspects and roles of glycoproteins can be found in many excellent textbooks and review articles such as the recently published second edition of *Essentials of Glycobiology* [1] and references therein.

It is with the significant advances made in rDNA technology over the last three decades that it became possible to produce large quantities of glycoproteins, some of which have been highly successful as human therapeutics. This is the case of erythropoiesis-stimulating agents (ESAs) that include the glycoproteins, erythropoietin-alpha (EPO-α), erythropoietin-beta (EPO-β), and darbepoetin-α (DPO-α), all of which are now widely used for the treatment of anemia associated with chronic kidney disease or cancer chemotherapy. Other significant examples of major glycoprotein therapeutics include follicle stimulating hormone (FSH) in the treatment of infertility, interferon beta-1a (IFNβ-1a) in the treatment of multiple sclerosis, and monoclonal antibodies (mAbs). In all of these cases, the glycan moiety plays a pivotal role in the biological activity. As such, techniques and methods capable of providing detailed information on glycosylation in glycoproteins have become widely applicable in both the manufacturing and regulatory sectors.

From an analytical point of view, the structural characterization of glycoproteins represents a major challenge as it involves not only issues related to the protein moiety (i.e., primary, secondary, or tertiary structural elements) but also with the microheterogeneity observed at glycosylation sites. For instance, glycan moieties vary in their composition, that is, the type of monosaccharide residues present; in their branching, that is, the degree of antennarity; or the site occupancy level. Thus, complete characterization is a major task and requires the use of multiple analytical techniques well suited to the particular aspect(s) being investigated. In addition, there are different strategies that can be used to characterize glycoproteins. For instance, the substrate may be subjected to partial or complete hydrolysis/proteolysis resulting in the generation of smaller moieties such as glycopeptides, glycans or individual monosaccharides and amino acids, each of which can then be analyzed separately. Alternatively, intact glycoproteins can be separated and studied using one of many suitable methods that have been developed with techniques such as high performance liquid chromatography (HPLC), mass spectrometry (MS) with or without prior separation, conventional gel electrophoresis, or capillary electrophoresis (CE). The latter technique has rapidly emerged as one of the most useful techniques for assessing intact glycoproteins due to its high resolving power, its capability for automation, its speed, the low

amounts required for analysis, and the quantitative results obtained. In the remainder, a brief overview of the state of the art on the use of CE for the analysis of intact glycoproteins will be provided. A more in-depth review of the field has been reported [2].

1.1 Overview on the Use of Capillary Electrophoresis for Intact Glycoproteins

There are three CE separation modes that have become particularly useful for the study of intact glycoproteins. These are capillary isoelectric focusing (CIEF), capillary electrophoresis sodium dodecylsulfate (CE-SDS), and capillary zone electrophoresis (CZE). Each mode will be briefly reviewed in the following sections.

1.1.1 Capillary Isoelectric Focusing (CIEF)

CIEF is a capillary alternative to slab gel isoelectric focusing (IEF), the traditional technique used for the determination of protein charge variants or for the determination of the isoelectric point (pI) of a protein. The basis of the separation involves the migration of a charged substrate in a pH gradient until it reaches its pI at which point it becomes neutral and stops migrating. The pH gradient within the capillary is generated using the same types of ampholytes as those used in slab gels. CIEF can be performed in one of two ways: as a two-step or as a one-step process. The two-step process involves an initial focusing of the protein in the gel-filled capillary followed by a final mobilization step that moves the protein band passed an online UV detection window. As a further refinement, the mobilization step can be eliminated entirely by using imaging CIEF (iCIEF) whereby the focused protein bands are detected by scanning the entire length of the capillary [3]. In the one-step process, both the focusing and mobilization steps occur concurrently upon the voltage being applied as a consequence of the presence of a residual electroosmotic flow (EOF) that moves the bands towards the detection window.

CIEF can be set up on any commercial CE instrument as no special equipment is required. Technical aspects of method development have been reviewed in details [2]. A CIEF starter kit for the two-step process is commercially available from AB Sciex (formerly the CE division of Beckman-Coulter). In iCIEF protein bands are detected over the entire length of the capillary and, as such, conventional CE instruments cannot be used. A specific commercial instrument is available from a single manufacturer, ProteinSimple (formerly Convergent Biosciences). Method development for iCIEF can be initiated with the commercial kit sold by the same company.

Kilar and Hjerten [4] were the first to report the use of CIEF to separate intact glycoproteins, the isoforms of transferrin (Tf). Since then, there have been many reports of other sialoglycoproteins to be characterized using that technique [2, 5, 6]. In these cases, the variable number of terminal sialic acid residues on glycan branches leads to overall charge differences and, consequently, to isoform separation. The generated isoform profile is often specific

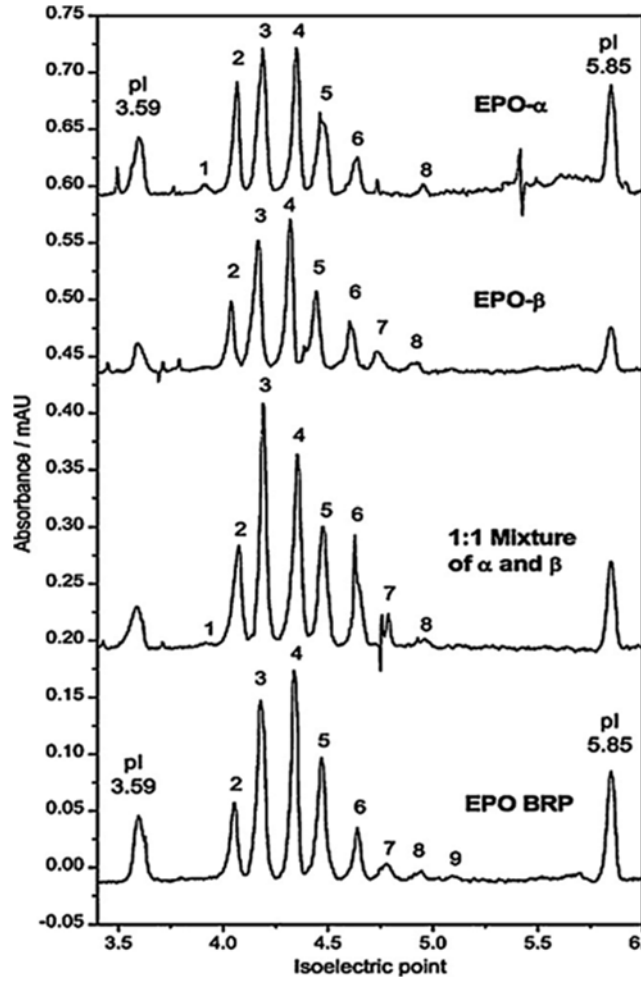

Fig. 1 Comparison of EPO samples by CIEF. Adapted from ref. [7] with permission

to a particular product as was well exemplified for EPO where EPO-α and EPO-β can be distinguished on the basis of their respective CIEF profiles (Fig. 1) [7].

Monoclonal antibodies (mAbs) are an important class of highly effective biopharmaceuticals for the treatment of cancer, autoimmune, and inflammatory diseases. While glycosylation represents only a small part of the total mass in mAbs, it plays a crucial role in the biological activity, particularly for the N-linked glycosylation site at Asn-297 of the CH2 domain of the heavy chain [8]. Glycosylation at other sites on the light chain is less common. In addition to variable sialylation on glycan chains as described above, mAbs may also feature charge variants due to deamidation at Asn residues and from variable C-terminal lysine cleavage [9]. The resulting complex charge heterogeneity profile can be characterized using CIEF or iCIEF [10]. These techniques can also provide fundamental information such as the isoelectric point (pI) of a

given substance or other critical information with regard to purity, stability, and consistency of manufacturing. Two recent studies by a group of international biopharmaceutical manufacturers have reported on the suitability of CIEF [11] and iCIEF [12] for the characterization of mAbs in support of process development and regulatory submissions in the biopharmaceutical industry.

1.1.2 Capillary Electrophoresis: Sodium Dodecylsulfate (CE-SDS)

Similarly to IEF, a capillary alternative to traditional slab gel SDS-PAGE, CE-SDS (also called SDS-CGE or CGE) has been developed. A review detailing technical aspects and applications has been recently published [13]. The methodology involves as a first step binding of SDS to proteins to a constant ratio leading to the formation of SDS-protein complexes with similar mass-to-charge ratios. Consequently, it is possible to separate the resulting negatively charged complexes solely on the basis of differences in hydrodynamic size. This is accomplished by using a capillary filled with a sieving matrix and applying the appropriate voltage to ensure migration towards the online UV detection window. The SDS complexes migrate according to their size, with the smaller components migrating faster. The protein complexes are typically detected online by UV absorbance at 220 nm. The method can be carried out under reducing or nonreducing conditions, both of which present advantages for product characterization. When compared to slab gel SDS-PAGE, CE-SDS provides increased resolution and is amenable to quantitation. For these reasons it has been widely adopted in the biopharmaceutical industry and applied at several stages of the manufacturing process such as in-process control, quality control, or lot release.

One of the main applications of CE-SDS is for the determination of protein size heterogeneity and, as such, it has proven particularly useful for the determination of the heavy chain glycan occupancy in mAbs [14, 15]. In such cases, the mAbs are reduced with dithiothreitol or 2-mercaptoethanol releasing the light chain, the heavy chain, and the nonglycosylated heavy chain. Along with the light chain, the nonglycosylated heavy chain can be resolved from the larger, glycosylated heavy chain (Fig. 2, top). The robustness of the methodology was examined through an international collaborative study involving industry and regulatory representatives and was found to be very good [16, 17]. Analysis of the unreduced mAb also provides useful information with regard to the presence of the nonglycosylated mAb as well as the presence of a number of fragments (Fig. 2, bottom). A commercial kit sold specifically for the purpose of IgG purity and heterogeneity determination is widely used and is available from AB Sciex.

1.1.3 Capillary Zone Electrophoresis (CZE)

In CZE charged molecules migrate in an open capillary filled with background electrolyte (BGE). While that could appear as a technically simpler separation mode than either CIEF or CE-SDS, method development is usually more demanding as the differential

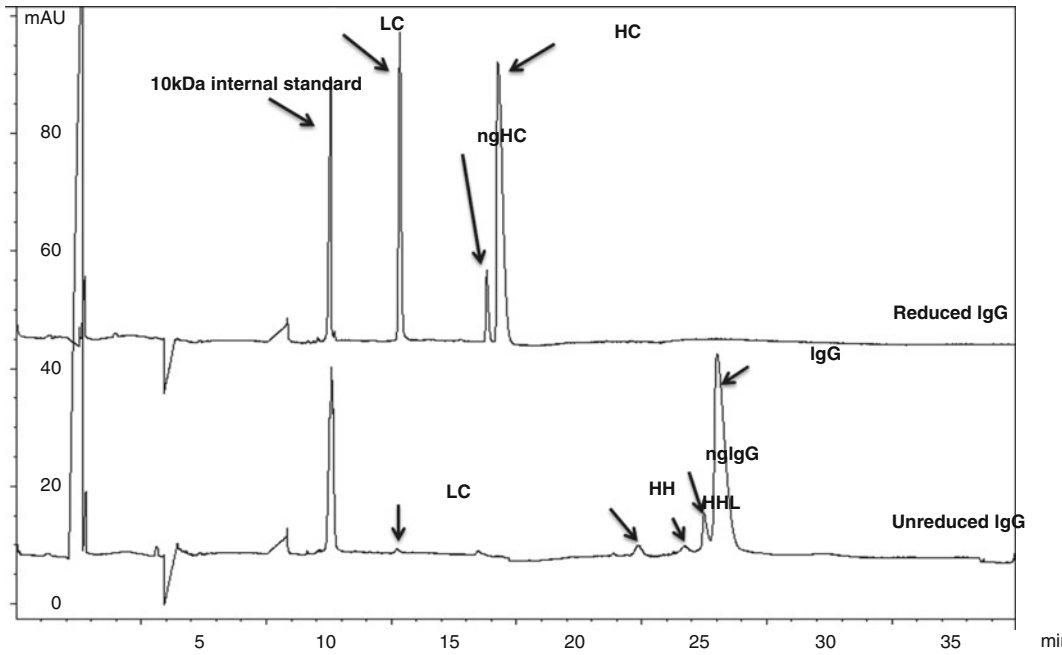

Fig. 2 Representative electropherograms of a reduced (*top*) and unreduced (*bottom*) IgG sample: *HC* heavy chain, *LC* light chain, *ngHC* nonglycosylated heavy chain, *IgG* IgG monomer, *ngIgG* nonglycosylated IgG monomer, *HHL* heavy-heavy-light, *HH* heavy-heavy. Peak assignment is based on Liu L.Y., Ratnayake C., Chapman J., Dontha N., Choo S., Reddy M.P. Beckman Coulter, Inc. Application Note A-1973A, 2003 (www.sciex.com/ce)

migration of substrates is dependent upon differences in overall charge-to-size ratios. For instance, it is just as possible to resolve substrates of similar sizes that differ in overall charge than to resolve substrates with similar overall charge that have different sizes. Thus, there are many more variables to consider in CZE method development among which are buffer type, ionic strength, pH, additives, or capillary coating. These methodological considerations have been reviewed elsewhere [2].

There are several detection systems that can be used with CZE. The conventional, online UV absorbance detection is widely used and provides sensitivity levels in the μg/mL range for most proteins with monitoring at 200 nm [2]. When increased sensitivity is required such as for the analysis of proteins in body fluids or tissues where analyte concentrations range from 10^{-10} to 10^{-13} M, fluorescence detectors are available. Laser-induced fluorescence (LIF) detection of both native proteins [18] and fluorescently labeled proteins [19] can be used and provides increased sensitivity of several orders of magnitude. Another detection mode that can be coupled to CZE is mass spectrometry (MS) [20]. However, volatile buffers and well-adapted capillary coating should be used to be compatible with MS.

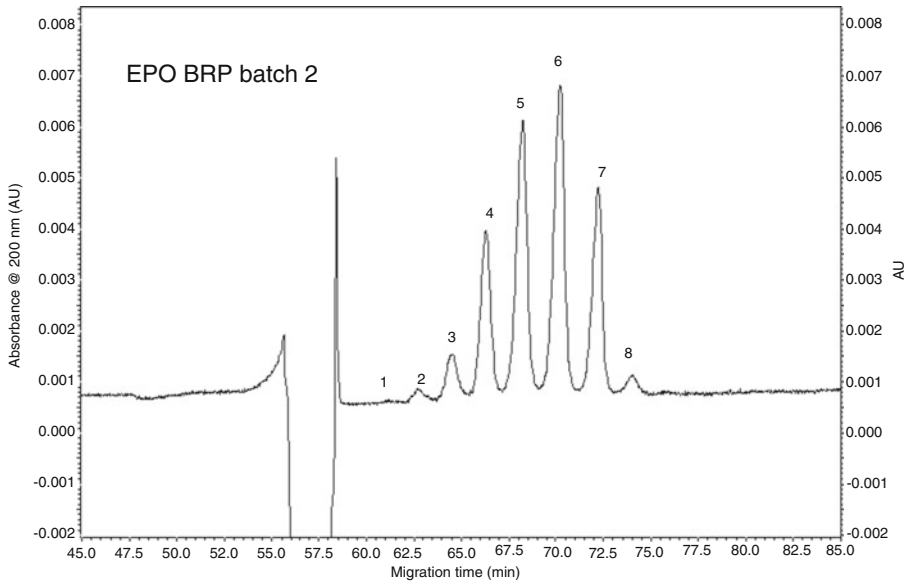

Fig. 3 Typical electropherogram of the European Pharmacopoeia Biological Reference Preparation for EPO, batch 2. Isoforms are numbered from 1 to 8 starting from the most basic to the most acidic. Conditions were as described in the monograph for EPO [21]

The use of CZE for the characterization of intact glycoproteins, particularly for the detection of sialoglycoprotein isoforms, has led to significant applications. For example, a CZE method for recombinant EPO is now used to replace conventional IEF as an identification test in the European Pharmacopoeia monograph for EPO concentrated solution [21]. The method separates the EPO biological reference preparation into eight isoforms (Fig. 3). Specifications on the distribution of glycoforms as a percentage of the total content in the test solution are provided and constitute an integral part of the identity test.

Several reports have also demonstrated that the determination of isoform profiles by CZE can be used in clinical settings. This is the case for the detection of carbohydrate-deficient transferrin (CDT), a biomarker of alcohol abuse [22]. Other sialoglycoproteins have also been examined in the context that differences in isoform profiles may enable prediction of disease state. Two such examples are α-acid glycoprotein [23, 24] and prostate-specific agent [25].

1.2 Analysis of Drug Products

Among other useful applications, CZE can be used to study final protein drug products. This aspect can be important from a quality point of view as it provides a way of assessing the structural integrity and/or stability of the active ingredient. It can also be used for comparison to other products in the context of evaluating biosimilars. However, the study of drug products entails consideration of

specific issues related to the content in active ingredient and the presence of excipients. Generally, a protein drug product contains low amounts of active ingredient, a situation that usually requires the addition of large amounts of excipients to maintain product stability or to prevent nonspecific adsorption to the container. Commonly used excipients vary widely in terms of their chemical nature and may include one or more substrates such as inorganic salts, amino acids, sugars, surfactants, or even other proteins such as human serum albumin (HSA).

One approach to the study of drug products is the removal of excipients. This was the case for a study of the marketed biopharmaceutical, Aranesp, where the removal of excipients was accomplished through ultracentrifugation, and enabled to detect significant differences of the isoform profile between product batches without seemingly affecting the biological activity [26]. Similarly, large amounts of HSA in a drug product could be removed by immunochromatography [27] for subsequent analysis by CZE. However, this type of approach requires careful attention to potential loss of product or the generation of product artifacts.

Another approach is to devise conditions that require no pretreatment of the product. This was the case for the development of a CZE method for the analysis of EPO drug formulations [28], which was later extended to other ESA drug products [29]. The ability of the method to resolve isoforms of the active ingredient in finished product enables the comparison of the isoform profile with that of the corresponding drug substance, allowing the assessment of the structural integrity and content of the active ingredients in finished products. The setup of the method is presented in the following sections.

2 Materials

2.1 Instrumentation and Setup

- Instrument: the method is carried out on a Beckman MDQ instrument fitted with a variable UV detector monitoring at 200 nm. The Beckman 32 Karat™ software is used for operation and data analysis (*see* **Note 1**). For different instruments, it may be necessary to adjust conditions in order to obtain similar results.
- Capillary: place an eCap Amine capillary, 50.2 cm total length × 50 μm i.d. (40 cm effective length) into a cassette with an 800 μm aperture and install the cassette into the CE instrument.

2.2 Background Electrolyte (BGE): 200 mM NaH_2PO_4, 1 mM $NiCl_2$, pH 4.0

- Prepare 500 mL of background electrolyte to a final concentration of 200 mM NaH_2PO_4/1 mM $NiCl_2$ by dissolving 13.8 g of $NaH_2PO_4 \cdot H_2O$ and 0.11885 g of $NiCl_2 \cdot 6H_2O$ in 400 mL doubly deionized H_2O (ddH_2O), adjust the pH to 4.0 with dilute acetic acid, and adjust the volume to 500 mL with ddH_2O (*see* **Note 2**).

- Store the BGE solution at 4–8 °C until required.
- Filter the required BGE aliquots with 0.45 μm Millipore filter disks prior to use.

2.3 Sample Preparation

- Keep frozen solutions of ESA drug substances (DS A-D) obtained from manufacturers in North America and Europe at −78 °C until required. Drug substances are: DS A (EPO-α), DS B (EPO-α), DS C (EPO-β), DS D (DPO-α).
- Drug products A-D are kept at 4–8 °C. Drug product A is a liquid formulation of EPO-α (10,000 IU/mL) with human serum albumin; Drug product B is a liquid formulation of EPO-α (10,000 IU/mL) with polysorbate 80; Drug product C is a lyophilized preparation of EPO-β (10,000 IU/vial) with polysorbate 20; Drug product D is a liquid formulation of DPO-α (0.20 mg/mL) with human serum albumin; Drug product D is a liquid formulation of DPO-α (0.50 mg/mL) with polysorbate 80. Drug products in liquid form are analyzed directly. Drug products in the form of lyophilized powder are first reconstituted with ddH_2O to the prescribed concentrations. Add aliquots of about 100 μL to micro-vials and place in the instrument sample storage area.
- Dissolve a content of a vial of the European Pharmacopoeia EPO Biological Reference Preparation, batch 2 containing 250 μg of EPO in 250 μL ddH_2O. For analysis using the EP method, desalt the sample according to the procedure in the monograph [21].

3 Methods

3.1 Capillary Cleaning, Conditioning, and Storage

3.1.1 Capillary Cleaning

- Carry out a capillary cleaning procedure for new capillaries or in situations requiring re-establishment of proper working conditions such as after long injection sequences (e.g. 50–60 injections) or prolonged storage periods (*see* **Note 3**).
- Apply the following sequence of rinses at a pressure of 20 psi in the forward mode: methanol for 5 min (for new capillaries or after a long storage period); 1 M HCl for 5 min; ddH_2O for 5 min; 1 M NaOH for 2 min; ddH_2O for 5 min; amine regenerator for 5 min (*see* **Note 4**).

3.1.2 Capillary Conditioning

- Apply the following sequence of rinses at a pressure of 20 psi in the forward mode for each rinse: 1 % w/v NaOH for 15 min; ddH_2O for 5 min; amine regenerator for 30 min; BGE-15 min (*see* **Note 5**).

3.1.3 Capillary Storage

For periods of less than 18 h, store the capillary by immersing the capillary ends in BGE. For longer periods, store the capillary by immersing the ends in ddH_2O and place the capillary at 2–8 °C in an upright position.

3.2 Analysis of ESA Samples

– Maintain the capillary at a temperature of 20 °C and the sample storage area at 10 °C.

– Perform the following sequence of pre-injection rinses: 0.1 M NaOH for 2 min at 20 psi; amine regenerator for 5 min at 20 psi; and BGE for 10 min at 20 psi.

– Inject the sample hydrodynamically for 8 s at 0.5 psi (*see* **Note 6**) and initiate the separation by applying a voltage of 8 kV at reverse polarity for 40 min (*see* **Note 7**). Data are collected at a wavelength of 200 nm.

– Use fresh BGE after six consecutive injections in order to avoid ion depletion [28] (*see* **Note 8**). Inject each sample in triplicate within a sequence.

Typical electropherograms of finished ESA products are shown in Fig. 4 along with those of their corresponding drug substances

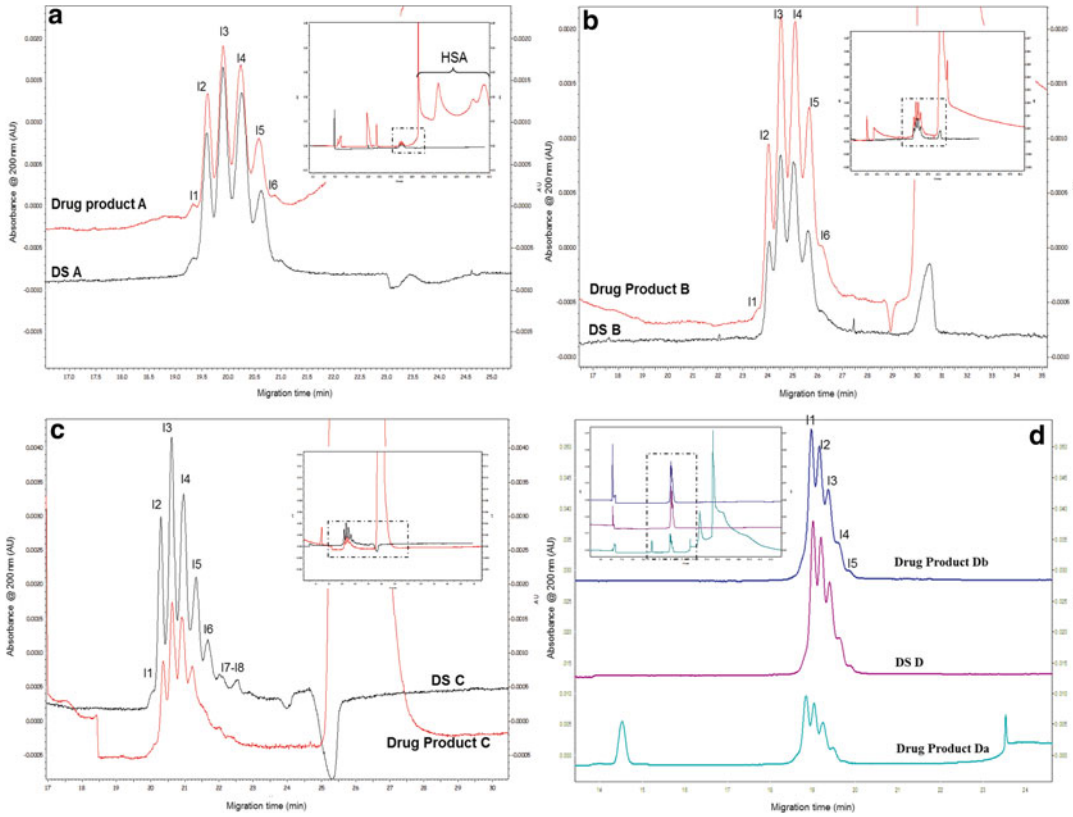

Fig. 4 Enlarged view of separated isoforms in electropherograms of finished ESA products: (**a**) EPO-α formulated with HSA; (**b**) EPO-α formulated with polysorbate 80; (**c**) EPO-β formulated with polysorbate 20; (**d**) DPO-α formulated with polysorbate 80 (*top*) and HSA (*bottom*). Electropherograms of corresponding drug substances (traces labeled DS **a**–**d**) are superimposed. Isoforms are labeled from the most acidic to the most basic. Insets: Complete electropherograms showing peaks due to excipients with *dashed rectangle* indicating enlarged area. Separation conditions: eCap Amine capillary; 200 mM sodium phosphate, 1 mM nickel chloride, pH 4.0; voltage: −8 kV [28, 29]

(traces labeled DS A-D). Under these separation conditions where the capillary walls are positively charged the more acidic substrates migrate ahead of the more basic ones (*see* **Note 9**). In addition, at pH 4.0, ESA products are negatively charged and migrate ahead of neutral and positively charged substrates. ESA products migrate before the major excipients. Isoforms are numbered from the most acidic to the most basic.

The EPO-α products (Fig. 4a, b) are each separated into 6 isoforms that include two minor isoforms, I1 and I6, and four major isoforms, I2-I5. The EPO-β drug substance, DS C (Fig. 4c), is separated into eight isoforms consisting of the three minor isoforms, I1, I7, and I8, and the five major isoforms, I2–I6. The analysis of DPO-α, DS D, (Fig. 4d) shows a profile indicative of the presence of multiple isoforms migrating more quickly than those from either EPO-α or EPO-β. The faster migration is expected and can be explained on the basis of the increased number of terminal sialic acids resulting from additional asparagine-linked glycans in DPO-α (*see* **Note 10**).

The ability of the method to resolve isoforms of each of the four active ingredients enables comparison of the isoform distribution of finished products with that of their respective drug substance. In general, finished products and their corresponding drug substances show similar isoform distribution despite variable formulations.

In conclusion, the CZE method described herein can be used for the monitoring of the active ingredient in ESA products no matter the type of formulation encountered (human serum albumin or polysorbate). In addition, the conditions provide a way to analyze products directly, that is, without pretreatment. This study demonstrates that capillary zone electrophoresis can be successfully applied to the analysis of most of the ESA products currently on the market in North America and Europe.

4 Notes

1. The method was originally developed on a PACE 5000 [28] and later transferred to a MDQ.
2. Recombinant EPO has a pI range of 4.4–5.4 and as such it is critical to adjust the BGE pH to a value close to 4.0 in order to prevent potential aggregation or precipitation.
3. The cleaning procedure should be used in moderation as it may significantly shorten the capillary lifespan if used too often. A capillary conditioning procedure is always performed after the cleaning procedure.
4. The amine regenerator solution requires to be warmed to room temperature and mixed well prior to use. The solution should be clear and colorless.

5. The capillary conditioning procedure is performed after the cleaning procedure, after a short storage period (3–10 days) or upon reduced capillary performance. Reduced capillary performance is attained when a %RSD value of greater than 10% for migration time is obtained for three consecutive injections.
6. Water dips are performed prior to and after analyte injection.
7. Under these conditions the generated current is about –32 μA and should be stable over the entire run time.
8. For injection sequences involving analysis of multiple samples it is desirable to prepare a sufficient number of BGE vials to carry out the entire sequence without interruption. Typically, a set of two BGE vials (one vial at the inlet and one vial at the outlet) will enable analysis of two samples (3 injections per sample).
9. The electroosmotic flow is in the direction of the anode. The electroosmotic mobility can be determined by injecting a neutral substance such as benzyl alcohol (data not shown).
10. The isoform separation of DPO-α is not optimal under the standard separation conditions at 200 mM phosphate. The resolution can be improved by increasing the BGE phosphate concentration to 300 mM [29].

References

1. Varki A, Cummings RD, Esko JD, Freeze HH, Stanley P, Bertozzi CR, Hart GW, Etzler ME (eds) (2009) Essentials of glycobiology, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
2. Girard M, Lacunza I, Diez-Masa JC, de Frutos M (2008) Glycoprotein analysis by capillary electrophoresis. In: Landers JP (ed) Handbook of capillary and microchip electrophoresis and associated microtechniques, 3rd edn. CRC Press Inc., Boca Raton, FL, pp 631–696, Chapter 22
3. Wu J, Pawliszyn J (1992) Universal detection for capillary isoelectric focusing without mobilization using concentration gradient imaging system. Anal Chem 64:224–227
4. Kilar F, Hjerten S (1989) Separation of the human transferrin isoforms by carrier-free high-performance zone electrophoresis and isoelectric focusing. J Chromatogr A 480:351–357
5. Wu J, Huang T, Pawliszyn J (2008) Isoelectric focusing in capillary systems. In: Landers JP (ed) Handbook of capillary and microchip electrophoresis and associated microtechniques, 3rd edn. CRC Press Inc., Boca Raton, FL, pp 563–579, Chapter 19
6. Haselberg R, de Jong GJ, Somsen GW (2011) Capillary electrophoresis-mass spectrometry for the analysis of intact proteins 2007-2010. Electrophoresis 32:66–82
7. Dou P, Liu Z, He JG, Xu JJ, Chen HY (2008) Rapid and high-resolution glycoform profiling of recombinant human erythropoietin by capillary isoelectric focusing with whole column imaging detection. J Chromatogr A 1190: 372–376
8. Anthony RM, Wermeling F, Ravetch JV (2012) Novel roles for the IgG Fc glycan. Ann NY Acad Sci 1253:170–180
9. Khawli LA, Goswami S, Hutchinson R et al (2010) Charge variants in IgG1. Isolation, characterization, in vitro binding properties and pharmacokinetics in rats. mAbs 2:613–624
10. Anderson CL, Wang Y, Rustandi RR (2012) Applications of imaged capillary isoelectric focussing technique in development of biopharmaceutical glycoprotein-based products. Electrophoresis 33:1538–1544
11. Salas-Solano O, Babu K, Park SS et al (2011) Intercompany study to evaluate the robustness of capillary isoelectric focusing technology for the analysis of monoclonal antibodies. Chromatographia 73:1137–1144

12. Salas-Solano O, Kennel B, Park SS et al (2012) Robustness of iCIEF methodology for the analysis of monoclonal antibodies: an interlaboratory study. J Sep Sci 35:3124–3129
13. Zhu Z, Lu JJ, Li S (2012) Protein separation by capillary gel electrophoresis: a review. Anal Chim Acta 709:21–31
14. Hunt G, Nashabeh W (1999) Capillary electrophoresis sodium dodecyl sulfate nongel sieving analysis of a therapeutic recombinant monoclonal antibody: a biotechnology perspective. Anal Chem 71:2390–2397
15. Rustandi RR, Washabaugh MW, Wang Y (2008) Applications of CE SDS gel in development of biopharmaceutical antibody-based products. Electrophoresis 29:3612–3620
16. Nunnally B, Park SS, Patel K et al (2006) A series of collaborations between various pharmaceutical companies and regulatory authorities concerning the analysis of biomolecules using capillary electrophoresis. Chromatographia 64: 359–368
17. Nunnally B, Park SS, Patel K et al (2007) A series of collaborations between various pharmaceutical companies and regulatory authorities concerning the analysis of biomolecules using capillary electrophoresis: additional instruments/buffer. Chromatographia 66:955–961
18. de Kort BJ, de Jong GJ, Somsen GW (2013) Native fluorescence detection of biomolecular and pharmaceutical compounds in capillary electrophoresis: detector designs, performance and applications: a review. Anal Chim Acta 766:13–33
19. García-Campaña AM, Taverna M, Fabre H (2007) LIF detection of peptides and proteins in CE. Electrophoresis 28:208–232
20. Pioch M, Bunz S-C, Neusüß C (2012) Capillary electrophoresis/mass spectrometry relevant to pharmaceutical and biotechnological applications. Electrophoresis 33:1517–1530
21. Erythropoietin concentrated solution 01/2008:1316, European pharmacopoeia, 7th edn. Published by EDQM, 01/2011
22. Delanghe JR, De Buyzere ML (2009) Carbohydrate deficient transferrin and forensic medicine. Clin Chim Acta 406:1–7
23. Lacunza I, Sanz J, Diez-Masa JC, de Frutos M (2006) CZE of human alpha-1-acid glycoprotein for qualitative and quantitative comparison of samples from different pathological conditions. Electrophoresis 27:4205–4214
24. Puerta A, Diez-Masa JC, Martin-Alvarez PJ et al (2011) Study of the capillary electrophoresis profile of intact α-1-acid glycoprotein isoforms as a biomarker of atherothrombosis. Analyst 136:816–822
25. Garrido-Medina R, Diez-Masa JC, de Frutos M (2013) On-capillary fluorescent labeling and capillary electrophoresis laser-induced fluorescence analysis of glycoforms of intact prostate-specific antigen. Electrophoresis 34:2295–2302
26. Schiestl M, Stangler T, Torella C et al (2011) Acceptable changes in quality attributes of glycosylated biopharmaceuticals. Nat Biotechnol 29:310–312
27. Lara-Quintanar P, Lacunza I, Sanz J et al (2007) Immunochromatographic removal of albumin in erythropoietin biopharmaceutical formulations for its analysis by capillary electrophoresis. J Chromatogr A 1153: 227–234
28. Bietlot HP, Girard M (1997) Analysis of recombinant human erythropoietin in drug formulations by high-performance capillary electrophoresis. J Chromatogr A 759: 177–184
29. Boucher S, Kane A, Girard M (2012) Qualitative and quantitative assessment of marketed erythropoiesis-stimulating agents by capillary electrophoresis. J Pharm Biomed Anal 71:207–213

Chapter 14

Quality Control of Therapeutic Monoclonal Antibodies at the Hospital After Their Compounding and Before Their Administration to Patients

Emmanuel Jaccoulet, Claire Smadja, and Myriam Taverna

Abstract

Monoclonal antibodies (mAbs) are widely used in cancer therapy and recently many new mAbs have gained EMA and FDA approvals for oncology indications. Here we describe a highly reproducible CZE method, relying on a cationic coating allowing separation and identification of a complex mixture of four compounded mAbs widely used in cancer therapy (cetuximab, rituximab, bevacizumab, and trastuzumab).

Key words Monoclonal antibodies, Capillary electrophoresis, Cationic coating, Polybrene, mAb identification, Quality control, Hospital

1 Introduction

Monoclonal antibodies (mAbs) have gained high interest in cancer therapy [1]. They concern a wide range of cancers such as leukemia, lymphoma, breast, colon, renal, or lung cancers [2, 3]. They offer many advantages such as more specific therapeutic action and low adverse effects [4]. Guidelines from manufacturers and health institutions state that mAbs available in concentrated form should be compounded under controlled and aseptic area in hospitals (also called production unit). Therefore, the main objectives of quality controls for these compounded products issued from the production unit are to identify and determine the mAb concentration before releasing and administrating them to patients. Qualitative as well as quantitative controls of these compounded drugs are of paramount importance for the quality, and the safety of use, and particularly to avoid medication errors. This is very challenging since the range of available mAbs, exhibiting similar physical and chemical properties, has increased while the compounding activity at the hospital is continuously increasing too. However, it is also worth noting that these mAbs have already

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obtained EMA and FDA approvals and thus do not require complex and extensive characterization such as micro-heterogeneity determination, degradation profile establishment, or impurity checking. A fast, simple, and rapid method allowing identification and quantitative determination represents a suitable quality control method for this purpose. Although quantitation is important, identification still remains the most challenging step in this QC of mAbs at the hospital.

Among the various analytical tools gathering these advantages, capillary electrophoresis (CE) meets the requirements of routine applications. CE of proteins has gained high interest in the field of quality control of biopharmaceutical industries [5–7] and is often used for the characterization of the mAbs [8]. However, proteins such as mAbs are prone to adsorption on silica capillary wall [9], which impacts the EOF and the migration times [10] and is detrimental for mAb identification. To avoid adsorption phenomenon, coating of the capillary is usually required [10]. Therefore, by employing a cationic polymer coating, polybrene, a reproducible separation of a complex mixture of four reconstituted mAbs (cetuximab, bevacizumab, trastuzumab, and rituximab) used at the hospitals has been obtained. Here, we describe the protocol of this rapid method which allows mAb identification based on their relative migration times.

2 Materials

All the solutions (buffers, SDS, perchlorate, polysorbate, polybrene), except therapeutic mAbs, should be prepared using ultrapure water (deionized) and analytical grade reagents. All the prepared solutions should be stored at 4 °C. Perchloric acid should be protected from the light. All the solutions and buffers should be filtered (0.2 μm filter) prior to analysis.

2.1 Infusion Bags Containing Monoclonal Antibodies

All the mAbs should be prepared according to the manufacturer's guidelines with 0.9% sodium chloride solution. The compounding should be done in a polyolefin bag for infusion after removing the desired volume of mAb (*see* **Note 1**). The preparation is executed under a laminar flow hood (*see* **Note 2**). The compounded monoclonal antibodies should be stored at 4 °C and protected from the light (*see* **Notes 3**).

2.2 Solution Preparation

1. 0.2% Polybrene coating solution: Weigh 200 mg hexadimethrine bromide (polybrene – M_W = 374 g/mol) and add 100 mL of water to reach a concentration of 0.2% polybrene. Mix gently, cover the glass jar with parafilm, and store at 4 °C.
2. Capillary washing solution: 50 mM SDS. Weigh 720 mg of SDS in an Amicon® conical plastic tube. Add 50 mL of water and mix. Store the 50 mM SDS solution at 4 °C. Prior to each analysis, warm the solution at ambient temperature (*see* **Note 4**).

3. 75 mM Phosphate buffer pH 3: Prepare 7.5 mL of 1 M phosphoric acid into a 100 mL graduated flask. Add 6.65 mL of 1 M NaOH (*see* **Note 5**). Mix gently. Add 95 mL of water. Adjust the pH to 3.0 ± 0.02 and complete with water to 100 mL.
4. 1 mM Perchloric acid solution: Add 4.3 µL of perchloric acid solution (70%) in a 50 mL volumetric flask and complete to 50 mL with water.
5. 67.5 mM Sodium phosphate/0.15 mM perchloric acid buffer pH 3.0: Add 7.5 mL of 1 mM perchloric acid to 42.5 mL of 75 mM phosphate buffer pH 3.0. The pH obtained should be at pH 3.0 ± 0.02.
6. 0.1% Polysorbate solution: Weigh 1.2 mg of polysorbate 80 in a conical tube. Add 12 mL of the sodium phosphate 67.5 mM/perchloric acid 0.15 mM buffer pH 3.0. Mix gently until the whole polysorbate is dissolved (*see* **Note 6**).
7. Running buffer: 67.5 mM Sodium phosphate/0.15 mM perchloric acid (0.1% polysorbate 80) pH 3.0. Transfer 2.5 mL of a 0.1% polysorbate 80 solution into a 25 mL volumetric flask. Complete with the 67.5 mM sodium phosphate/0.15 mM perchloric acid pH 3.0 and mix.
8. Glutamate internal standard: Weigh 19 mg of glutamate. Dissolve in 10 mL of water in a conical tube.

3 Methods

3.1 Sample Preparation

1. For each Mab infusion bag: Mix gently the infusion bags (1–2 min) at room temperature before collecting a small volume (*see* **Note 7**).
2. Mix equal volumes of each of the collected solutions (4 mAbs) in 15 mL Amicon® conical tube (*see* **Note 8**).
3. Add 170 µL of the mAb mixture to 30 µL of the glutamate internal standard in a 200 µL conical insert (*see* **Note 9**).

3.2 Capillary Pretreatment

1. Place an uncoated capillary: 50 µm I.D. × 360 µm O.D., total length of 60.5 cm, 50 cm effective length into the CE cartridge.
2. Rinse the uncoated capillary with water (5 min, 20 psi), NaOH 1 N (*see* **Note 5**) (8 min, 20 psi), and water (5 min, 20 psi).

3.3 CZE Analysis

1. Prior to the analysis, rinse the capillary for 2 min with water, followed by 2 min with 50 mM SDS at 20 psi, 2 min with water (20 psi), and finally 1 min with water (20 psi) (*see* **Notes 10** and **11**).
2. Then rinse with NaOH 0.1 N (*see* **Note 12**) at 20 psi followed by 0.2% polybrene coating solution for 2 min at 20 psi.

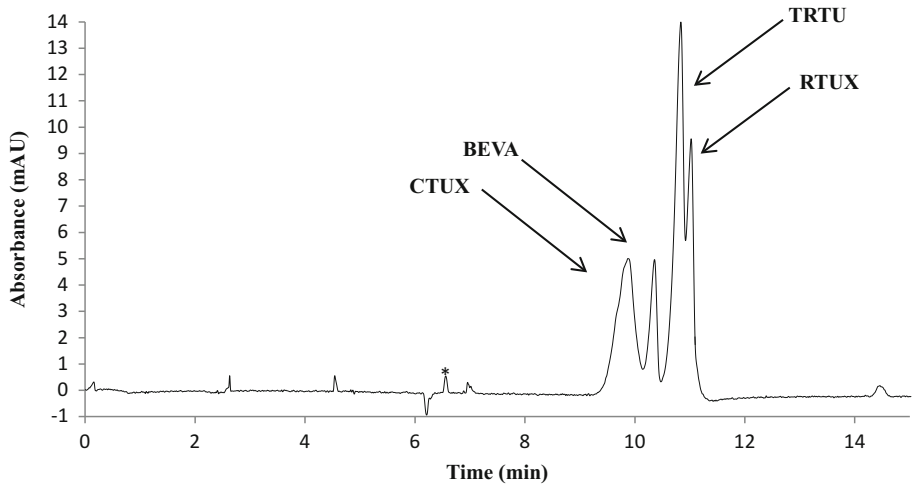

Fig. 1 CZE profile of the mAb mixture with polybrene coating (0.2 %). *Glutamine (IS). The four major peaks are migrating with the following order: cetuximab (CTUX), bevacizumab (BEVA), trastuzumab (TRTU), and rituximab (RTUX). BGE: 67.5 mM Phosphate buffer, 0.15 mM perchloric acid, 0.01 % Tween 80 pH 3; applied voltage: −20 kV; injection (0.5 psi, 10 s). UV detection 214 nm. Capillary length (50 μm I.D.; effective length 50 cm)

3. Rinse with the running buffer for 3 min at 20 psi.
4. Inject the samples hydrodynamically at 0.5 psi for 10 s. Then soak the capillary for a few seconds in water to wash it.
5. Apply a negative voltage (−20 kV) between the vials containing the running buffer (*see* **Note 13**). Select the UV detection at 214 nm.
6. Rinse the capillary after the separation for 5 min at 20 psi with NaOH 0.1 N and then water for 1 min.

3.4 CE Data Analysis

CE run corresponding to the mixture of four mAbs (cetuximab, bevacizumab, trastuzumab, and rituximab) and the IS gives an electropherogram with one major and minor peaks (Fig. 1) (*see* **Note 14**).

1. Integrate the peaks using the CE software to measure migration times of both IS and mAb peaks.
2. Calculate the RMT from the ratio of the migration time (MT) of each mAb to the one of IS (mAb Mt/IS Mt) (*see* **Note 15**).

4 Notes

1. Wear gloves and glasses when handling those products. In order to avoid any water leak from the bag, insertion and removal should be done through the injection port of the bag. A luer-lock

syringe connected to a 18-gauge needle should be used. mAbs are separately compounded (i.e., one bag for one mAb).

2. The main objective of this step is to limit contamination such as bacterial one. In addition, mAbs are easily prone to degradation. If no specific caution is taken when handling the product, some degradations may occur and change physical or chemical properties of mAbs leading to poor repeatability of the method.
3. Light and temperature may promote mAb degradation.
4. SDS solutions should be warmed prior to their use as SDS may precipitate at low temperatures.
5. The NaOH 1 N employed is commercial.
6. Polysorbate is a viscous solution. To prepare 0.01% polysorbate solution, make successive dilutions.
7. Use a new syringe and needle for each collection.
8. Most of the mAbs are compatible with polyethylene (PE), polyvinyl chloride (PVC), or polyolefin (POE). Check the material compatibility prior to CE analysis.
9. Vials for CE analysis should be carefully filled according to CE apparatus guidelines. In Beckman Coulter MDQ, a conical insert of 200 μL is needed to be placed in a specific vial for injection.
10. This step should be systematically applied after protein analysis. We found that SDS 50 mM is suitable for rinsing capillary before protein analysis.
11. We found that using two different rinses (2 min + 1 min) with water (with two different vials of water) is better for limiting SDS contamination of the 0.1 N NaOH vial used for capillary pre-coating.
12. NaOH 0.1 N was obtained by diluting the commercial NaOH 1 N with water.
13. The separation is carried out under a negative voltage since the coating is cationic and the mAbs are positively charged under the acidic BGE. Such repulsive conditions are necessary to avoid protein adsorption and to have a good resolution and reproducibility.
14. Minor peaks correspond to excipients of the mAbs except the IS peak. IS peak is expected to migrate at 6.5–6.7 min. If the localization of the IS is difficult, one should increase the amount of IS in the mixture while decreasing mAb volume.
15. The calculation should be done for each mAb in triplicate. The RMT of each mAb is specific and the difference between each RMT is statistically significant.

References

1. Reichert JM (2014) Antibodies to watch in 2014: mid-year update. MAbs 6:799–802
2. Berger M, Shankar V, Vafai A (2002) Therapeutic applications of monoclonal antibodies. Am J Med Sci 324:14–30
3. Reichert JM (2011) Antibody-based therapeutics to watch in 2011. MAbs 3:76–99
4. Glassman PM, Balthasar JP (2014) Mechanistic considerations for the use of monoclonal antibodies for cancer therapy. Cancer Biol Med 11:20–33
5. Wätzig H, Günter S (2003) Capillary electrophoresis-a high performance analytical separation technique. Clin Chem Lab Med 41:724–738
6. Kamoda S, Kakehi K (2011) Evaluation of glycosylation for quality assurance of antibody pharmaceuticals by capillary electrophoresis. Electrophoresis 29:3595–3604
7. Righetti PG, Candiano G (2011) Recent advances in electrophoretic techniques for the characterization of protein biomolecules: a poker of aces. J Chromatogr A 1218: 8727–8737
8. Creamer JS, Oborny NJ, Lunte SM (2014) Recent advances in the analysis of therapeutic proteins by capillary and microchip electrophoresis. Anal Methods Adv Methods Appl 6:5427–5449
9. Stutz H (2009) Protein attachment onto silica surfaces-a survey of molecular fundamentals, resulting effects and novel preventive strategies in CE. Electrophoresis 30:2032–2061
10. Lucy CA, MacDonald AM, Gulcev MD (2008) Non-covalent capillary coatings for protein separations in capillary electrophoresis. J Chromatogr A 1184:81–105

Chapter 15

Capillary Electrophoresis-Ultraviolet-Mass Spectrometry (CE-UV-MS) for the Simultaneous Determination and Quantification of Insulin Formulations

Julie Schappler and Serge Rudaz

Abstract

This chapter describes a CE-UV-MS method for the identification and quantification of insulin in pharmaceutical formulations in a single run. The CE conditions are optimized to avoid the adsorption of the protein onto the capillary wall. Particular attention is paid regarding the choice of the internal standard. A strategy based on multiple injections is applied to correct both ionization and injection variabilities. The methodology is validated according to international guidelines and the obtained accuracy profile demonstrates the ability of the CE-UV-MS method to quantify insulin in pharmaceutical formulations within a ±5 % acceptance range. This strategy can be implemented in the field of quality control, as well as in the detection of counterfeits.

Key words Capillary electrophoresis, Identification, Insulin, Intact protein, Multiple injections, Quality control, quantification, Mass spectrometry

Abbreviations

ACN	Acetonitrile
BGE	Background electrolyte
CE	Capillary electrophoresis
ddH_2O	Double-distilled water
EOF	Electroosmotic flow
ESI	Electrospray ionization
FS	Fused silica
I.D.	Internal diameter
ICH	International Conference of Harmonization
iPrOH	Isopropanol
LOD	Limit of detection
m/z	Mass-to-charge ratio
MeOH	Methanol
MS	Mass spectrometry

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RSD	Relative standard deviation
S/N	Signal-to-noise ratio
SFSTP	Société Française des Sciences et Techniques Pharmaceutiques
TIE	Total ion electropherogram
TOF	Time-of-flight
UV	Ultraviolet
XIE	Extracted ion electropherogram

1 Introduction

The amount of recombinant proteins produced by biotechnology has grown considerably in the last few years [1]. During the biopharmaceutical development and production processes, numerous parameters are needed for regulatory purposes, regarding the identity, quantity, quality, and purity of the products. Since unofficial channels exist to obtain these products without prescription or without extensive evidence for quality control, the analysis of therapeutic proteins is also relevant for the parallel market and counterfeit drugs. Therefore, it is essential to have analytical methods that quickly monitor the identity, quality, and quantity of these biopharmaceuticals.

For the identification and quantification of protein formulations, the analysis of proteins in their intact form is a promising approach because no tedious sample preparation, such as a digestion step, is required [2]. In order to perform the simultaneous identification and quantification of an intact protein, whether in a pharmaceutical formulation or in another matrix, the hyphenation of capillary electrophoresis (CE) and mass spectrometry (MS) *via* an electrospray ionization (ESI) source appears as an attractive option [3]. CE offers high speed, great efficiency, and low solvent and sample consumptions, while MS provides selectivity, sensitivity, and identification features. Due to its high mass range and mass accuracy, the time-of-flight (TOF) analyzer is particularly well suited for the detection of multi-charged macromolecules when ESI is employed [4].

In this study, a CE-UV-TOF/MS method is developed for the analysis of formulations of recombinant human insulin. Patients suffering from diabetes mellitus often buy insulin online without prescription due to the potentially lower cost, which increases the risk of finding counterfeit drugs in the parallel market. In the context of public health, CE coupled to UV-TOF/MS for quality control of these biopharmaceutical formulations offers nice features with simultaneous identification and quantification of insulin, thanks to an external calibration approach. A multiple-injection technique, used to get both information in one single run, is selected and consists of the successive injection of a reference standard of insulin and the unknown sample. The complete methodology is fully validated

according to the guidelines of the International Conference of Harmonization (ICH) and can be further applied to pharmaceutical formulations obtained in drugstores and on the Web without a formal prescription [5].

2 Materials

2.1 Apparatus and Material

1. Experiments are carried out with an HP ^{3D}CE system from Agilent Technologies (Waldbronn, Germany), equipped with an on-capillary diode array detector, an autosampler, and a power supply able to deliver up to 30 kV. The instrument is coupled to a 6210 LC/MS TOF mass spectrometer (Agilent Technologies, Palo Alto, CA, USA) via a coaxial sheath flow ESI interface from Agilent.
2. Uncoated fused silica (FS) capillaries are obtained from BGB Analytik AG (Böckten, Switzerland), with an inner diameter (I.D.) of 50 μm, a total length of 80 cm, and an effective length of 22 cm for UV detection.

2.2 Chemical, Reagent, and Sample Solutions

2.2.1 Solutions for Capillary Electrophoresis-Ultraviolet-Mass Spectrometry (CE-UV-MS)

1. Background electrolyte (BGE): Prepare 50 mL of a mixture of ammonium formate 75 mM (pH 9.0)-ACN (90:10, *v/v*) (*see* **Note 1**).
   In a beaker, insert 500 μL of ammonia 28% and add ddH_2O up to 40 mL. Adjust the pH to 9.0 with formic acid 99% and add ddH_2O up to 50 mL. Take 40 mL of this solution and add 10 mL of acetonitrile (ACN). This solution can be kept for 1 month at 4 °C.
2. Sheath liquid: Prepare 100 mL of a mixture of iPrOH-ddH_2O-formic acid (50:50:1, *v/v/v*).
   In a beaker, insert 50 mL of isopropanol (iprOH) and 50 mL of ddH_2O and add 1 mL of formic acid 99%.

2.2.2 Solutions of Insulin Formulations for the Validation of the CE-UV-MS Procedure

1. Solutions used for calibration (calibration standards): Prepare three independent samples (0.5 mL) of insulin at 5 U.I./mL. Mix 25 μL of insulin at 100 U.I./mL with 250 μL procaine at 100 μg/mL. Add 225 μL ddH_2O (*see* **Note 2**).
2. Solutions used for validation (validation standards): Prepare nine independent samples (0.5 mL) of insulin at 3.6 U.I./mL. Mix 18 μL of insulin at 100 U.I./mL with 250 μL procaine at 100 μg/mL. Add 232 μL ddH_2O (*see* **Notes 2** and **3**).
3. Solutions used for validation (validation standards): Prepare nine independent samples (0.5 mL) of insulin at 5 U.I./mL. Mix 25 μL of insulin at 100 U.I./mL with 250 μL procaine at 100 μg/mL. Add 225 μL ddH_2O (*see* **Notes 2** and **3**).

4. Solutions used for validation (validation standards): Prepare nine independent samples (0.5 mL) of insulin at 6.4 U.I./mL. Mix 32 µL of insulin at 100 U.I./mL with 250 µL procaine at 100 µg/mL. Add 218 µL ddH_2O (*see* **Notes 2** and **3**).

2.2.3 Solutions of Insulin Formulations for the Routine Use of the CE-UV-MS Procedure

1. Solutions to be quantified (unknown samples): Prepare two independent samples (0.5 mL) of the insulin formulation at 5 U.I./mL.
   Mix 25 µL of the insulin formulation at 100 U.I./mL with 250 µL procaine at 100 µg/mL. Add 225 µL ddH_2O (*see* **Notes 4** and **5**).

3 Methods

3.1 Capillary Electrophoresis-Ultraviolet-Mass Spectrometry (CE-UV-MS) (See Note 6)

1. New capillaries are pretreated by applying a pressure of 1 bar to the capillary inlet and using the following sequence: MeOH, 1 M HCl, ddH_2O, 0.1 M NaOH, ddH_2O, and BGE for 5 min each.
2. The capillary is thermostated at 25 °C and the samples maintained at ambient temperature in the sample tray.
3. The BGE is prepared daily and renewed every three runs.
4. The in-between rinsing run is carried out by pumping fresh BGE through the capillary at 2 bar for 1 min.
5. When the capillary is not in use, it is rinsed with ddH_2O and dry-stored at room temperature.
6. The sample is introduced into the capillary by hydrodynamic injection using the following sequence: the first sample is injected at 50 mbar for 10 s (equivalent to 0.68% of the total capillary length). Then, a plug of BGE is injected at 50 mbar for 130 s (8.90% of the total capillary length). The second sample is injected at 50 mbar for 10 s (0.68% of the total capillary length). Finally, a plug of BGE is injected at 50 mbar for 4 s (0.27% of the total capillary length) (Table 1, *see* **Notes 7–10**).
7. The separations are carried out at 30 kV with positive polarity at the inlet and an initial ramping voltage of 5000 V/s.
8. Insulin and procaine are detected with UV detection at 195 nm.
9. The sheath liquid is delivered at a flow rate of 4 µL/min by a syringe pump system.
10. The ESI source parameters are set: the ESI voltage at +4500 V, the nebulizing gas pressure at 4 psi, the drying gas flow rate and temperature at 4 L/min and 150 °C, respectively, and the fragmentor voltage at 400 V.

Table 1
Sequence of the multiple-injection approach (*see* Notes 7 and 10)

Injection plug	Composition	Sample name	Injection conditions
1	Insulin 5 U.I./mL + procaine 50 µg/mL	Calibration standard	50 mbar × 10 s
	BGE	BGE	50 mbar × 130 s
2	Insulin 3.6–6.4 U.I./mL + procaine 50 µg/mL Or Insulin 5 U.I./mL + procaine 50 µg/mL	Validation standard (in validation procedure) Or Unknown sample (in routine analysis)	50 mbar × 10 s
	BGE	BGE	50 mbar × 4 s

11. One spectrum is acquired *per* second (9742 transients/spectrum) in the 900–2500 *m/z* range.
12. The CE-ESI-MS total ion electropherogram (TIE) shows two peaks (Fig. 1a, Table 2): the first peak (a) corresponds to the insulin standard from the first injected sample, and the second peak (b) is insulin from the second injected sample to be tested. The $[M+3H]^{3+}$ and $[M+4H]^{4+}$ ions are detected as the major extracted ions (1937 and 1453 *m/z*, respectively, Fig. 1b) on the mass spectrum. The extracted ion electropherogram (XIE) is obtained using both ions (Fig. 1c) and integration achieved on the XIE.
13. The CE-UV electropherogram shows six peaks (Fig. 1d, Table 2): the first three peaks (c, *, e) come from the first injected sample, and the three last peaks (d, **, f) come from the second injected sample. Peaks c and d are procaine migrating before the electroosmotic flow (EOF), peaks * and ** are neutral excipients detected in the EOF (e.g., metacresol and glycerol), and peaks e and f are insulin migrating after the EOF.

3.2 Analysis of Insulin Formulations by CE-UV-MS: Validation of the Procedure

1. Evaluate the method selectivity by comparing typical electropherograms obtained by injecting ddH_2O (CAL 00), procaine at 50 µg/mL in ddH_2O (CAL 0), and a validation standard (VS). No interference should be observed at the migration time corresponding to the procaine and insulin peaks in UV or MS measurements (Fig. 2).
2. Inject the first sample (calibration standard) and then the second sample (validation standard) according to the injection sequence (Table 1), and perform the CE-UV-MS analysis.

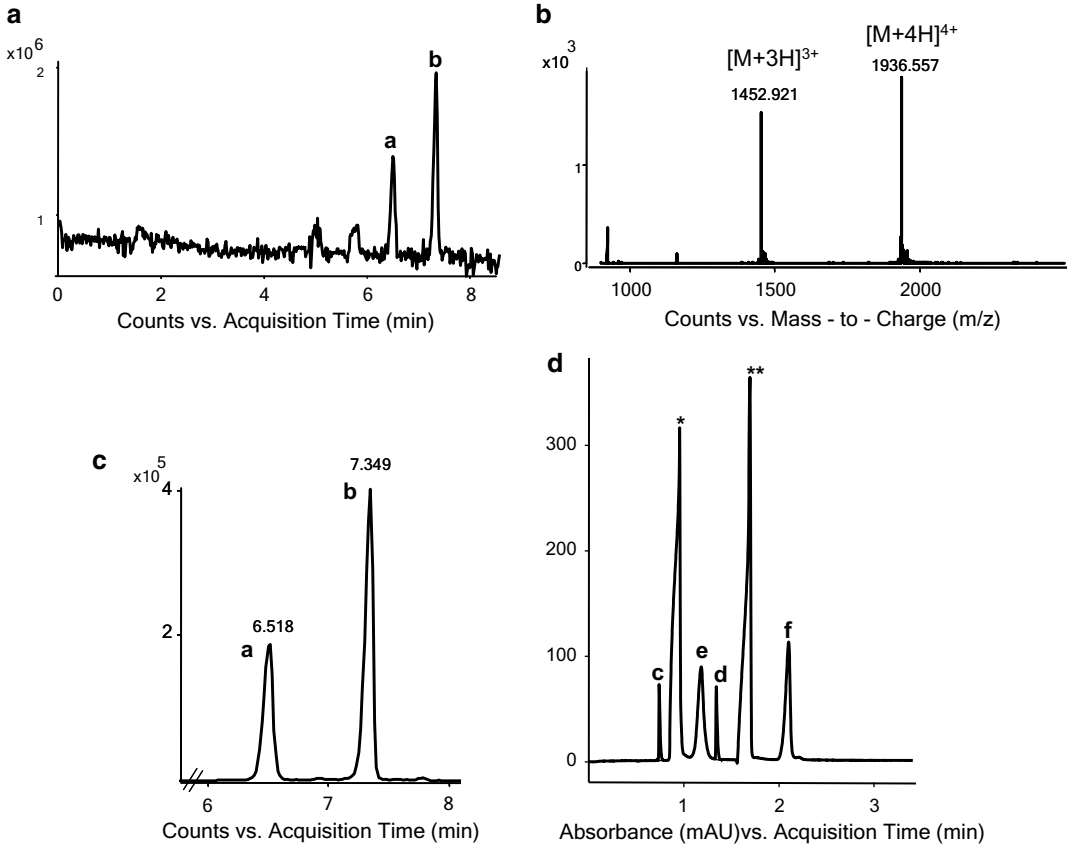

Fig. 1 Analysis of insulin by CE-UV-MS. (**a**) MS detection, total ion electropherogram (TIE), (**b**) MS detection, extracted mass spectrum, (**c**) MS detection, extracted ion electropherogram (XIE), and (**d**) UV detection. (a) and (e) insulin at 3 U.I./mL from the first injection, (b) and (f) insulin at 5 U.I./mL from the second injection, (c) procaine at 50 μg/mL from the first injection, (d) procaine at 50 μg/mL from the second injection, *neutral excipients from the first injection, and ** neutral excipients from the second injection. Adapted from [5], with permission

Table 2
Correspondence between injected samples and detected peaks

	Detected peaks			
Injected samples	**ESI-MS**	**UV**		
1	a Insulin	c Procaine	* Excipients	e Insulin
2	b Insulin	d Procaine	** Excipients	f Insulin

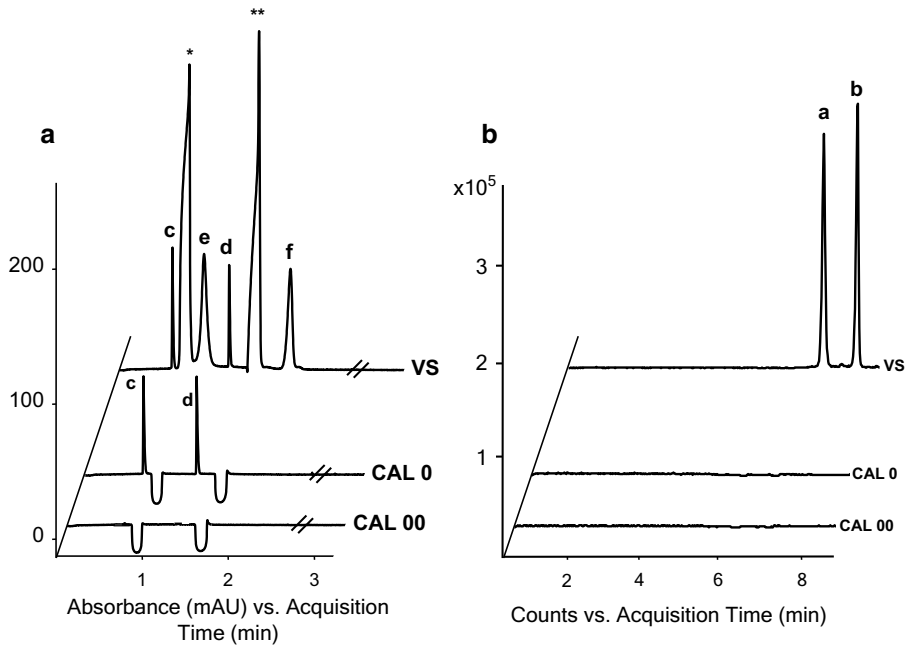

Fig. 2 Selectivity of the CE-UV-MS method. (**a**) UV detection and (**b**) MS detection. Injection of: ddH_2O (CAL 00), procaine at 50 μg/mL (CAL 0), and validation standard (VS) at 6.4 U.I./mL. (a) and (e) insulin at 3 U.I./mL from the first injection, (b) and (f) insulin at 5 U.I./mL from the second injection, (c) procaine at 50 μg/mL from the first injection, (d) procaine at 50 μg/mL from the second injection, * neutral excipients from the first injection, and ** neutral excipients from the second injection. Adapted from [5], with permission

Repeat the analysis with the three independent replicates ($n = 3$), the three levels of concentration ($k = 3$), and the three series ($j = 3$) (*see* **Notes 3** and **11**).

3. Correct peak area as follows: The area of insulin peak 2 detected by MS (corresponding to peak b in Fig. 1c) is corrected by the area of the procaine peak 2 detected by UV (peak d in Fig. 1d). The same correction is done for the area of insulin peak 1 detected by MS (peak a over peak c). The ratio $(b/d)/(a/c)$ is used for trueness and precision calculations (*see* **Note 12**).
4. Estimate the method trueness for each concentration level (expressed as recovery, %, obtained by the ratio of calculated concentration over true concentration).
5. Estimate the method precision by the variances of repeatability and intermediate precision (expressed as RSD, %), obtained by variance decomposition (ANOVA).
6. Construct the accuracy profile (Fig. 3). The upper and lower confidence limits (plain outer lines, respectively, in Fig. 3) are calculated from the trueness (plain central line in Fig. 3) and represent the total error of the method, based on the confidence interval ($\alpha = 5\,\%$) (*see* **Note 13**).

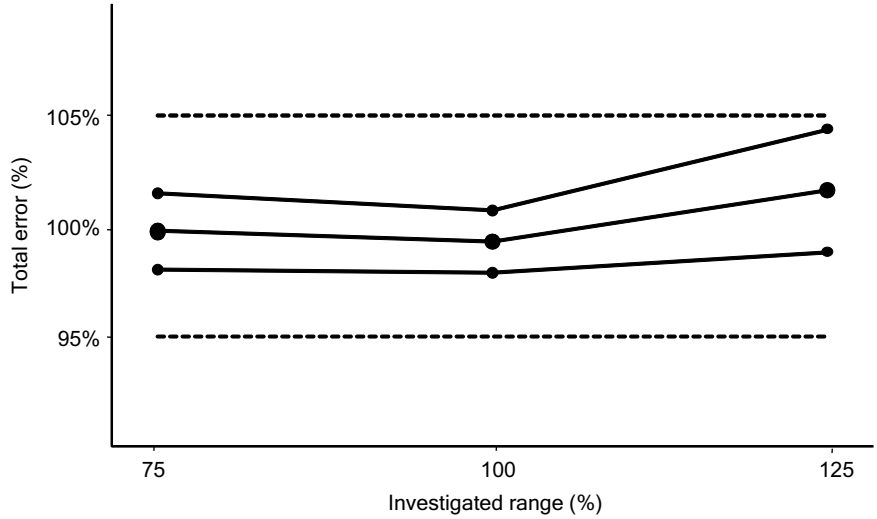

Fig. 3 Accuracy profile of the CE-UV-MS method ($j=3$, $k=3$, $n=3$). (___) plain central line represents the method trueness, (__) plain outer lines represent the method confidence limits, and (---) dotted lines represent the method acceptance limits. Adapted from [5], with permission

7. Make a decision: If the upper and lower confidence limits are included into the acceptance limits (±5%, dotted lines in Fig. 3), the CE-UV-MS method can be considered valid for insulin determination over the investigated concentration range (3.6–6.4 U.I./mL). Quantification of real samples can be performed with an appropriate method uncertainty.

3.3 Analysis of Insulin Formulations by CE-UV-MS: Routine Use of the Procedure

1. Inject the first sample (calibration standard) and then the second sample (unknown sample) according to the injection sequence (Table 1), and perform the CE-UV-MS analysis. Repeat the analysis with the second preparation of the unknown sample.
2. Identify insulin by comparing the mass spectrum of peak a and peak b (Fig. 4c). $[M+3H]^{3+}$ and $[M+4H]^{4+}$ ions should be detected as the major extracted ions (1937 and 1453 *m/z*, respectively).
3. Correct peak area as follows: The area of the unknown peak detected by MS (corresponding to peak b in Fig. 4b) is corrected by the area of the procaine peak detected by UV (corresponding to peak d in Fig. 4a). The same correction is done for the area of insulin calibration standard detected by MS (peak a over peak c).
4. Calculate the concentration of insulin: The mean ratio (b/d)/(a/c) is used for quantification, expressed as a relative concentration ± confidence interval [%] (*see* **Note 14**).

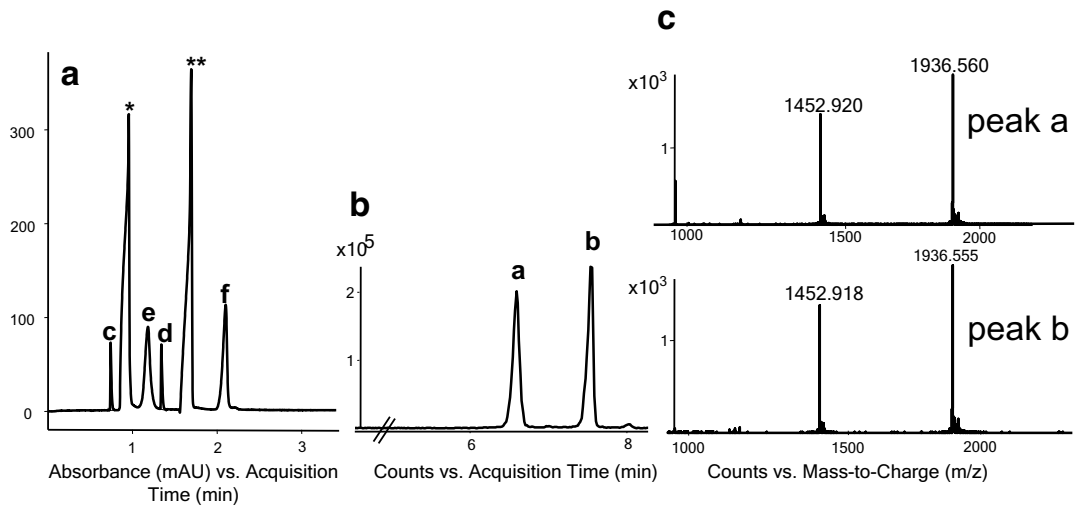

Fig. 4 Analysis of an insulin formulation purchased from the Web by CE-UV-MS. (**a**) UV detection, (**b**) MS detection, extracted ion electropherogram (XIE), and (**c**) MS detection, extracted mass spectra. Adapted from [5], with permission

5. Make a decision: The unknown sample can be considered compliant with the expected specifications if its identity is confirmed and its concentration falls within the expected limits (±5 % around the target value) (*see* **Note 15**).

4 Notes

1. Volatile BGE has to be used to be directly ESI-MS compatible. ACN is added to improve CE performance and reduce the adsorption of the protein onto the capillary wall [6].
2. The exact nature and concentration of the excipients of insulin formulations remain unknown in most cases; for instance, the zinc and glycerine quantities are not mentioned in the manufacturer's datasheet of Actrapid®, hindering the reconstitution of the formulation. To overcome this issue, a reference batch of Actrapid® at 100 U.I./mL (Novo Nordisk A/S, Bagsvaerd, Denmark) is used as a stock solution for the calibration and validation samples.
3. According to ICH guidelines, as well as recommendations from the "Société Française des Sciences et Techniques Pharmaceutiques" (SFSTP), three series ($j = 3$) of three independent replicates ($n = 3$) are prepared at each concentration level ($k = 3$) [7].
4. If the formulation to be quantified is not 100 U.I./mL, the dilution should be adapted so that a sample at 5 U.I./mL is analyzed.

5. Two independent samples are prepared since it has been demonstrated that most of the variability comes from the repeatability.
6. Under these conditions, the limit of detection of the method (LOD, estimated for a S/N = 3) is 0.2 U.I./mL, while the response function is linear over a concentration range of 0.2–7 U.I./mL.
7. The unknown sample is injected with the second injection plug, so the sample to be quantified is less subject to variability caused by the previous hydrodynamic injections of the sample "calibration standard" and BGE.
8. In CE-ESI-MS, the ionization standard should exhibit similar ionization behavior as that of the protein to be quantified. As neither stable isotopically labeled nor structural analogues are easily available, a standard of insulin at a known concentration is first injected (calibration standard), followed by an injection of the sample to be quantified (validation standard or unknown sample) in the same run. This procedure allows decreasing the run-to-run variability of the ionization. Short-term variability of the ionization process can still occur since both peaks do not co-migrate.
9. In addition to the ionization standard, an injection standard is added to both samples to correct for the variability of the hydrodynamic injection between both injections in the same run. Procaine is selected because it migrates before the EOF and UV detection is used to avoid any additional source of ionization variability.
10. With a BGE plug corresponding to approximately 9% of the capillary length between both injections, the procaine peaks are sufficiently resolved to be easily integrated.
11. The double role of the first injected sample (calibration sample and internal standard) improves the throughput of the validation process, resulting in fewer injections.
12. A linear response function without significant intercept is used as calibration function.
13. The accuracy profile is selected as the decision tool to evaluate the method's capacity to quantify samples over the expected concentration range (75–125%, i.e., 3.6–6.4 U.I./mL). The accuracy takes into account the total error of the method and includes the combination of systematic (trueness) and random (precision) errors. The accuracy profile is constructed by first reporting the trueness at each concentration level (plain central line in Fig. 3). Then the upper and lower confidence limits (UL and LL, respectively, plain outer lines in Fig. 3) are calculated at each concentration level with the following equations and reported on the graph:

$$UTL = trueness + t_{df,\alpha}\sqrt{s_R^2}$$

$$LTL = trueness - t_{df,\alpha}\sqrt{s_R^2}$$

where $t_{df,\alpha}$ is the Student constant and s_R^2 is the intermediate precision variance determined thanks to the regular ANOVA-based variance decomposition.

14. The confidence interval associated to the result mean ($\bar{x}$) is expressed as

$$\bar{x} = t_{df,\alpha}\sqrt{\frac{s_r^2}{N} + s_g^2}$$

where $t_{df,\alpha}$ is the Student constant, N is the number of analyses performed during routine analysis for each sample, and s_r^2 and s_g^2 are the repeatability and the inter-day variances, respectively, determined during validation thanks to the regular ANOVA-based variance decomposition.

15. The example is provided with a sample of insulin purchased on the Web. A concentration of $100.2 \pm 2.2\%$ is obtained for this sample, which is considered compliant.

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References

1. Ledford H (2007) Biotechs go generic: the same but different. Nature 449:274–276
2. Staub A, Guillarme D, Schappler J, Veuthey JL, Rudaz S (2011) Intact protein analysis in the biopharmaceutical field. J Pharm Biomed Anal 55:810–822
3. Julie Schappler, Jean-Luc Veuthey, and Serge Rudaz (2008) Coupling CE and Microchip-Based Devices with Mass Spectrometry. In: Satinder Ahuja and M. Ilias Jimidar (eds), Separation Science and Technology, Vol 9, Capillary Electrophoresis Methods for Pharmaceutical Analysis, Academic Press: The Netherlands, pp 477–521. ISBN: 978-0-12-372573-8
4. Staub A, Schappler J, Rudaz S, Veuthey JL (2009) CE-TOF/MS: fundamental concepts, instrumental considerations and applications. Electrophoresis 30:1610–1623
5. Staub S, Rudaz S, Veuthey JL, Schappler J (2010) Multiple injection technique for the determination and quantitation of insulin formulations by capillary electrophoresis and time-of-flight mass spectrometry. J Chromatogr A 1217:8041–8047
6. Staub A, Comte S, Rudaz S, Veuthey JL, Schappler J (2010) Use of organic solvent to prevent protein adsorption in CE-MS experiments. Electrophoresis 31:3326–3333
7. Hubert P, Nguyen-Huu JJ, Boulanger B, Chapuzet E, Chiap P et al (2004) Harmonization of strategies for the validation of quantitative analytical procedures: a SFSTP proposal—part I. J Pharm Biomed Anal 36:579–586

Chapter 16

Applications of an Automated and Quantitative CE-Based Size and Charge Western Blot for Therapeutic Proteins and Vaccines

Richard R. Rustandi, Melissa Hamm, Catherine Lancaster, and John W. Loughney

Abstract

Capillary Electrophoresis (CE) is a versatile and indispensable analytical tool that can be applied to characterize proteins. In recent years, labor-intensive SDS-PAGE and IEF slab gels have been replaced with CE-SDS (CGE) and CE-IEF methods, respectively, in the biopharmaceutical industry. These two CE-based methods are now an industry standard and are an expectation of the regulatory agencies for biologics characterization. Another important and traditional slab gel technique is the western blot, which detects proteins using immuno-specific reagents after SDS-PAGE separation. This technique is widely used across industrial and academic laboratories, but it is very laborious, manual, time-consuming, and only semi-quantitative. Here, we describe the applications of a relatively new CE-based western blot technology which is automated, fast, and quantitative. We have used this technology for both charge- and size-based CE westerns to analyze biotherapeutic and vaccine products. The size-based capillary western can be used for fast antibody screening, clone selection, product titer, identity, and degradation while the charge-based capillary western can be used to study product charge heterogeneity. Examples using this technology for monoclonal antibody (mAb), Enbrel, CRM197, and *Clostridium difficile (C. difficile)* vaccine proteins are presented here to demonstrate the utility of the capillary western techniques. Details of sample preparation and experimental conditions for each capillary western mode are described in this chapter.

Key words Capillary western, Western blot, Mab, *Clostridium difficile* vaccine, CRM197

1 Introduction

Since the introduction of the western blot by Towbin and Renart [1, 2], it has become one of the most important methods in protein characterization, especially in complex cell lysates. The use of the western blot has increased tremendously in many laboratories worldwide over the last 30 years. The western blot can be considered as a pseudo two-dimensional analytical technique where in the first dimension protein is separated by either size or charge using gel electrophoresis and in the second dimension the

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separated protein of interest is immuno-blotted. During this immuno-blot the protein of interest is probed using a specific antibody (primary) and detected by a labeled secondary antibody against the Fc-region of the primary antibody. The secondary antibody is usually labeled with an enzyme such as horseradish peroxidase (HRP) or alkaline phosphatase (AP) or other fluorescence agents [3]. Even after three decades, traditional westerns are still performed manually, are labor intensive, only moderately reproducible, and qualitative or semi-quantitative at best [4, 5].

In recent years, CE has emerged as an indispensable tool to analyze proteins due to its high resolving power, quantitative nature, relative speed, and the potential to automate. One of the many contributions of CE in the biopharmaceutical industry is the replacement of traditional, labor-intensive SDS-PAGE (size) and IEF (charge) slab gel electrophoresis by CE-SDS (CGE) and CE-IEF methods, respectively [6–13]. In 2006 the first capillary IEF-based western was introduced [14] and subsequently in 2011 the capillary SDS-PAGE-based western was introduced with the first commercial instrument called Simon™ from ProteinSimple [15, 16]. Over a 3-year period, several versions of this technology have come out with various names: Sally, Peggy, Sally Sue, Peggy Sue, and Wes, all collectively called Simple Western. Simple Western technology is a size and charge CE-based western blot system that provides advantages of being fully automated in operation, except for the sample preparation, quantitative and reproducible with CV <10% [16, 17]. Technical details of the Simple Western technology have been described in several publications [15–18]. Briefly, proteins are separated in a capillary by either molecular weight (size) or pI (charge). The capillaries contain a proprietary UV-induced cross-linked chemical that covalently binds proteins to the wall of the capillary after exposure to UV light. After proteins are bound, the capillary is washed to remove the separation matrix and each capillary is incubated with the target-specific primary antibody followed by the species-specific secondary HRP-labeled antibody. Lastly the capillaries are incubated with a luminol and peroxide mixture to develop the chemiluminescence signal which is then recorded with a charge-coupled device (CCD) camera across all capillaries. All the above operations are fully automated including the electropherogram signal integration. We have recently evaluated this western technology for qualitative and quantitative analysis of vaccine protein components, residual bovine serum albumin (BSA), dot blot for 15-valent pneumococcal conjugate vaccine, and enzymatic activity for PARP-1 [16, 19–21]. Here we provide detailed protocols for the Simple Western method and examples of its application such as clone selections, rapid identity test, heat stress stability, and acidic variants biological activity in vaccine and therapeutic proteins. A monoclonal antibody, a heavily sialylated Fc-fusion glycoprotein for joint inflammation pain (Enbrel), CRM197, and four toxin proteins from *C. difficile* vaccine will be discussed.

2 Materials

2.1 Size-Based Western

1. Sally, Sally Sue, Peggy, Peggy Sue, Wes (ProteinSimple) (*see* **Note 1**).
2. UV-activated clear Teflon coated bare fuse silica capillary 5 cm long with100 μm ID and 375 OD μm (ProteinSimple) (*see* **Note 2**).
3. SDS-containing separation matrix (ProteinSimple) (*see* **Note 3**).
4. Stacking matrix (ProteinSimple) (*see* **Note 3**).
5. Running buffer (ProteinSimple) (*see* **Note 3** and **Note 4**).
6. Matrix removal buffer (ProteinSimple) (*see* **Note 4**).
7. Concentrated wash buffer (ProteinSimple) (*see* **Note 4**).
8. Luminol S (ProteinSimple) (*see* **Note 5**).
9. Peroxide (ProteinSimple) (*see* **Note 5**).
10. Sample buffer 10× (ProteinSimple) (*see* **Note 5**).
11. Streptavidin HRP (ProteinSimple or Jackson Immuno Research) (*see* **Note 5**).
12. Goat anti-rabbit HRP (ProteinSimple) (*see* **Note 6**).
13. Goat anti-mouse HRP (ProteinSimple) (*see* **Note 6**).
14. Antibody Diluent (ProteinSimple) (*see* **Note 7**).
15. Fluorescent standard markers (ProteinSimple) (*see* **Note 8**).
16. Biotinylated molecular weight markers (ProteinSimple) (*see* **Note 9**).
17. D,L-Dithiothreitol (DTT).
18. Iodoacetamide.
19. 20% SDS solution.

2.1.1 Master Mix (2×) Reducing Solution Preparation (Final Volume 120 μL) (See Note 10)

1. One vial lyophilized fluorescent protein markers, 2% SDS, 2× sample buffer, 200 mM DTT.
2. Vortex and place at 22–25 °C for up to 1 day only.

2.1.2 Molecular Weight Standards Preparation (Final Volume 20 μL) (See Note 9)

1. One vial lyophilized biotinylated molecular weight markers, 1× sample buffer, 100 mM DTT.
2. Vortex until all dissolved.
3. Place in 90 °C heating block for 10 min.
4. Equilibrate at room temperature for 10 min and centrifuge it to collect all liquid.
5. Transfer 5 μL of sample into a 384-well plate, avoid bubbles while transferring.
6. Store remaining MW markers at −70 °C for up to 1 year.

2.1.3 Reducing Condition Sample Preparation

1. Place 4 µL sample in 500 µL Eppendorf Tube.
2. Add 4 µL of 2× prepared master mix solution.
3. Place in 90 °C heating block for 10 min (*see* **Note 11**).
4. Equilibrate at room temperature for 10 min and centrifuge it to collect all liquid.
5. Transfer 5 µL of sample into 384-well plate, avoid bubbles while transferring.

2.2 Charge-Based Western

1. NanoPro 1000, Peggy, and Peggy Sue (ProteinSimple) (*see* **Note 12**).
2. UV-activated clear Teflon coated bare fuse silica capillary 5 cm long with 100 µm ID and 375 µm OD (ProteinSimple) (*see* **Note 2**).
3. Anolyte (100 mM NaOH).
4. Catholyte (10 mM Phosphoric acid).
5. Wash Concentrate (ProteinSimple).
6. Pharmalyte Ampholyte, pH 5–8 (GE Healthcare).
7. Pharmalyte Ampholyte, pH 8–10.5 (GE Healthcare).
8. Luminol (ProteinSimple) (*see* **Note 13**).
9. Peroxide XDR (ProteinSimple) (*see* **Note 13**).
10. Bicine Chaps (ProteinSimple) (*see* **Note 14**).
11. Antibody Diluent (ProteinSimple).
12. pI Standard Ladder 1 (ProteinSimple) (*see* **Note 15**).
13. pI Standard Ladder 3 (ProteinSimple) (*see* **Note 15**).
14. pI Standard 8.4 (ProteinSimple) (*see* **Note 16**).
15. pI Standard 9.7 (ProteinSimple) *see* **Note 16**).
16. Premix Ampholyte Free (ProteinSimple) (*see* **Note 17**).
17. Premix Ampholyte 3–10, G2 (ProteinSimple) (*see* **Note 17**).
18. Premix Ampholyte 4–7, G2 (ProteinSimple) (*see* **Note 17**).
19. Premix Ampholyte 4–9, G2 (ProteinSimple) (*see* **Note 17**).

2.2.1 Preparation of Premix

1. Combine 1000 µL premix ampholyte of pI range that is appropriate with 60 µL pI standard ladder (*see* **Note 18**).
2. Vortex thoroughly.

2.2.2 Preparation of Antibodies

1. Dilute primary antibody in antibody diluent as appropriate (*see* **Note** 19).
2. Dilute secondary antibody in diluent as appropriate (*see* **Note** 19).

2.2.3 Sample Preparation

1. Dilute samples in bicine chaps buffer to the predetermined concentration within the linear range (*see* **Note 20**).

2. Urea may also be added to the sample for a denatured condition.
3. Mix premix and sample in a 3:1 volumetric ratio (*see* **Note** 21).
4. Vortex thoroughly (*see* **Note** 21).

3 Methods

3.1 Size-Based Western

The size-based Western blot is performed under denaturing and reducing or nonreducing conditions. The conditions provide the advantage of less matrix interference when analyzing proteins in crude cell lysate/culture compared to standard ELISA which is run under native conditions. Furthermore, the protein identification in a size-based western is much more specific due to the additional dimension of separation by molecular weight compared to ELISA which is one dimension (batch mode). Here we present first the general analysis protocol and then demonstrate several important applications for the use of this automated and quantitative capillary size-based western technology in vaccine and therapeutic protein development.

1. Prepare a sample plate containing luminol S/peroxide, primary antibody, secondary antibody, antibody diluent, stacking matrix, separation matrix, running buffer, and samples according to individual instrument user's manual.
2. Perform self-test check (*see* **Note 22**).
3. Prepare Simple Western instrument parameters according each system user's manual (see **Note 23**).
4. Click run.
5. Wait till the fluorescent image appears indicating samples are injected.
6. Analyze data using Compass software (*see* **Note 24**).
7. If results are not satisfactory, then perform CE and western optimizations
8. Optimize standard CE parameters (*see* **Note 25**).
9. Optimize standard western blot parameters if necessary (*see* **Note 26**).

The first example of the utility of this technique is in antibody reagent screening (Fig. 1a) and clone screening (Fig. 1b). Figure 1a illustrates antibody screening for toxin B (TcdB), one of the four protein antigens from a *C. difficile* vaccine [22], which has MW ~270 kDa (*see* **Note 27**). Ten different monoclonal antibodies obtained commercially (lane 1–10) are screened for the best response. The antibody in lane 7 clearly has the best signal among all antibodies screened and therefore is chosen for further use in

this capillary western. Another antibody screening example for a different protein, CRM197 (MW ~58 kDa), which is a nontoxic form of diphtheria toxin used as a carrier protein in many polysaccharide conjugate vaccines [23], is also shown in Fig. 1a. Two antibodies (mAb1 and mAb2) are evaluated against CRM197 and the figure shows that mAb1 also detects CRM197 fragment (p35) that is different from that detected using mAb2 (CRM197 fragment

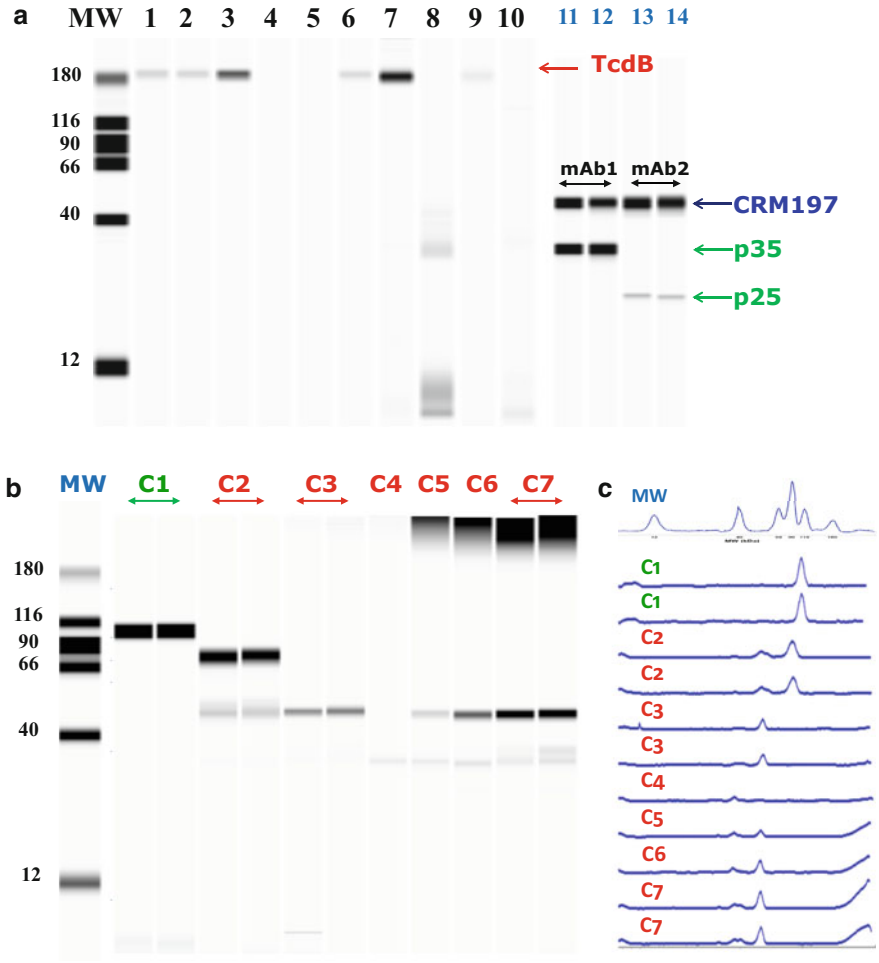

Fig. 1 (**a**) Virtual gel image of ten antibodies screened for TcdB (43 μg/mL, MW ~270 kDa) in lanes 1–10. All antibodies were obtained from different commercial sources. The antibody in lane 7 provides the strongest response and was subsequently used for Western blot assays. In order to observe the high MW protein band, the stacking and sample loading times were adjusted from default conditions to 16 and 8 s, respectively. Similarly, two different mAbs were evaluated for CRM197 (50 μg/mL, MW 58 kDa) in lane 11–14. Each mAb was tested in duplicate. Both mAbs detect the main peak of CRM197 but they are specific for different epitopes as demonstrated by the two different CRM197 fragments (p35 and p25) observed. All antibodies used were from mouse with 1:25 dilution; goat anti-mouse HRP was used as the detection secondary antibody. (**b**) Virtual gel image from proCDTb protein clone selection experiment in cell culture indicating clones that produce significantly different protein product (C1 is intended, while other clones (C2–C7) provide either fragments and/or aggregates, no protein). ProCDTb-specific rabbit pAb with 1:1000 dilution was used. (**c**) The actual electropherograms of (**b**)

(p25)). Hence these two antibodies detect two different epitopes on CRM197 and both become very useful for CRM197 degradation characterization. The antibody screening process can be completed in one run using the Simple Western platform that can take as little as 3 h, while with a manual western this would take at least 1–3 days and significant manual labor. Clone selection is commonly performed on a small scale and the quantity available for testing at this stage is often low. Therefore we need a sensitive assay to screen for the best product quality and titer. Simple Western is an ideal method for this purpose as it requires low sample volume (5–10 μL), sensitivity is high, and results are obtained quickly compared to traditional methods. Figure 1b demonstrates the use of this technology for clone selection for binary toxin B (proCDTb), another one of the four protein antigens from the *C. difficile* vaccine which has MW ~95 kDa. Seven different clones obtained from *Baculovirus* constructs (C1-C7) are screened to determine which clone produced the highest yield and best quality. Clone 1 shows the intended product quality while clone 2 and 3 produce degradation products with no intact proCDTb. Clone 4 shows no product at all, while clones 5, 6, and 7 have increasing aggregation and degradation products. Therefore, clone 1 is chosen to move forward for development.

The second example showing the usefulness of this technology is for identity testing and antigen stability in the final drug product of our tetravalent *C. difficile* vaccine. Figure 2 shows the identity test using Simple Western for the final drug product containing four antigens in our *C. difficile* vaccine. TcdA and TcdB have MW ~308 and 270 kDa, respectively. Both large MW proteins are not resolved using ProteinSimple gel matrix; consequently they appear above the highest MW standard of 180 kDa (*see* **Note 27**) but the MW is not accurate. The binary toxins, proCDTb and CDTa, appear at their respective MW ~95 and 48 kDa. This result demonstrates that Simple Western technology can perform identity analysis in the *C. difficile* vaccine drug product containing four different proteins potentially replacing the manual western blot.

Additionally we have used this technology for assessing heat-stressed stability of our *C. difficile* vaccine samples. The samples are tested by both Simple Western and ELISA. ELISA is the typical method used as a stability indicating assay for final drug product but the results are sometimes difficult to understand and they can be misleading due to the one-dimensional nature of the assay. Western blots have the potential of providing further information about each antigen's stability because of the additional dimension of size or charge separation. Figure 3 illustrates the use of the size-based simple western to generate stability profiles of individual antigens in control and heat-stressed (37 °C) final container *C. difficile* vaccine. Both TcdA and TcdB aggregate upon stress, indicated by the appearance of high MW bands, while CDTa and proCDTb are very stable. The slight decrease in proCDTb

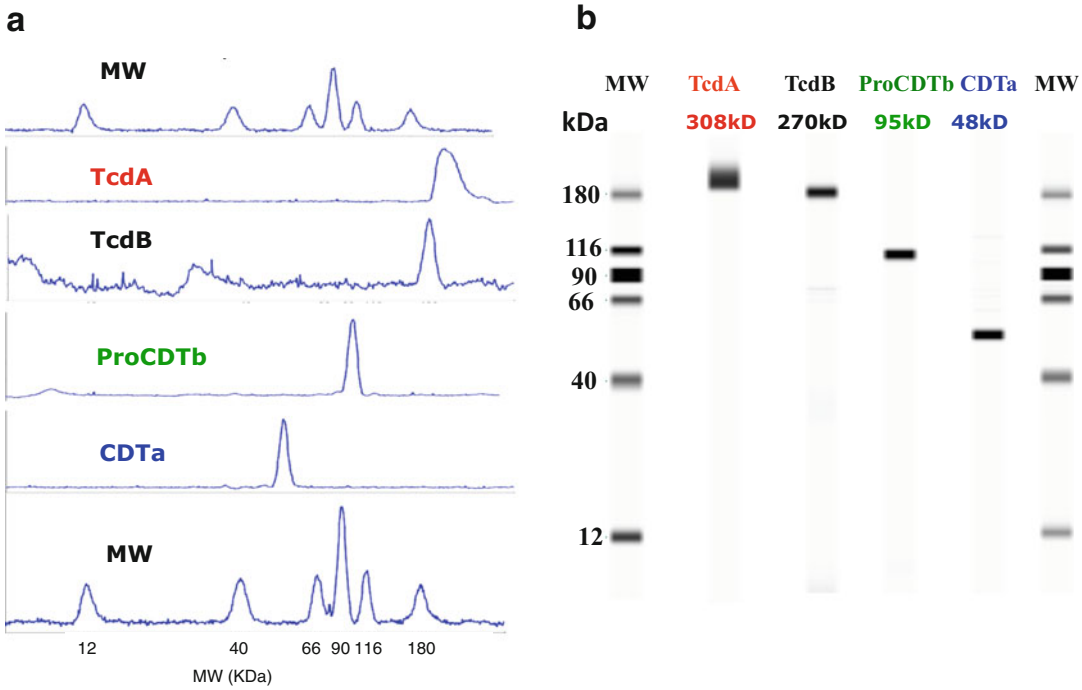

Fig. 2 Identity test for tetravalent *C. difficile* vaccine drug product using rabbit pAb (1:1000 dilution) specific for TcdA, TcdB, CDTa, and proCDTb with final concentration of 5, 2.5, 1.4, and 1.4 μg/mL, respectively (*see* **Note 27**). (**a**) Electropherograms and (**b**) virtual gel image

intensity at 37 °C is attributed to sample preparation. Hence, the capillary western blot provides an extra layer of information about stability by detecting both degradation and aggregation products as well as a quantifiable mass.

The third example where this technology has been applied is in measuring titer for early cell culture samples. We use Simple Western for concentration measurements in both binary toxin CDTa (*C. difficile* vaccine) and for a therapeutic monoclonal antibody. Figure 4 shows typical CDTa electropherograms for titer optimization in various cell culture conditions. The quantitation is done by comparing with purified standard CDTa from 0.45 to 7.5 μg/mL. Furthermore CDTa concentrations measured by Simple Western correlate well with RP-HPLC [16, 24] (the original method which is used before Simple Western exists, Fig. 4c). Monoclonal antibody upstream production and quality can also be monitored by this Simple Western as depicted in Fig. 5. Electropherograms and virtual images by Simple Western are shown for mAb production from day 6 through day 14. This result indicates an increase in measured mAb (IgG) over time under the nonreducing condition and also confirms good quality (no fragments; only HC and LC) by reducing western. This capillary western can be used as alternative method to the standard protein A HPLC for measuring IgG production.

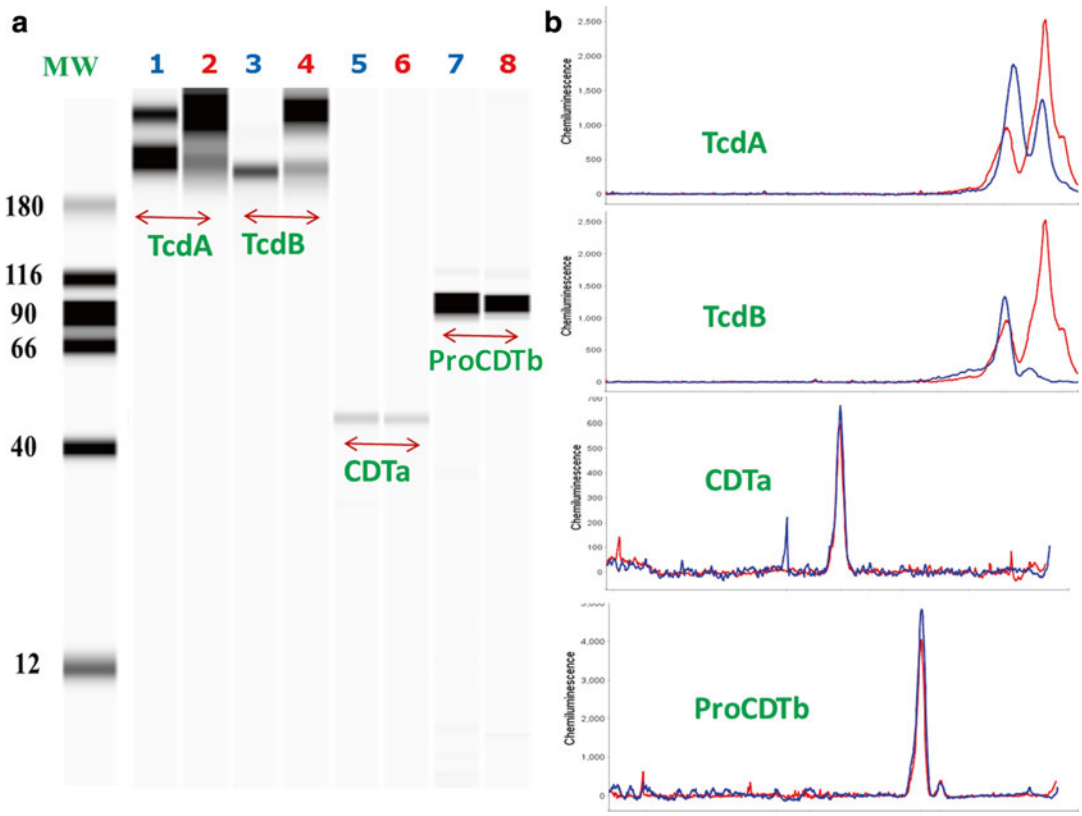

Fig. 3 Stability indicating assay for tetravalent *C. difficile* vaccine drug product. (**a**) The virtual gel image comparing drug product at −70 °C (*blue*) and 14 days at 37 °C (*red*), TcdA (lane 1 vs. 2), TcdB (lane 3 vs. 4), CDTa (lane 5 vs. 6), and proCDTb (lane 7 vs. 8). (**b**) The electropherograms of 3A. Note that TcdA initially has some high MW aggregation that increases at 37 °C for 14 days, while TcdB has no aggregation initially but becomes aggregated upon heat stress. The aggregation intensity is in most cases much higher than monomer in an immuno-type of assay; hence the decrease in monomer intensity is not proportional to the increase in aggregation intensity. No clipping or aggregation was observed for CDTa and proCDTb. A slightly lower intensity observed for proCDTb peak at 37 °C was attributed to sample preparation (see also Fig. 6 for charge-based western data)

3.2 Charge-Based Western

The charge-based western separates proteins by their pIs in a pH gradient matrix (capillary IEF) and the pI-separated protein is blotted in a similar manner as in standard size-based western blots. This experiment can be performed either native or denatured (by adding various concentrations of urea). Although the charge-based western is seldom used, it can provide complementary information to the size-based western (*see* **Note 28**). Here we present first a detailed protocol of the method and provide several examples of how this charge-based technology can be a useful tool; as a potential stability indicating method for multivalent vaccine products, as a method for evaluating biosimilar candidates, and in evaluating mAb charge heterogeneity in cell culture.

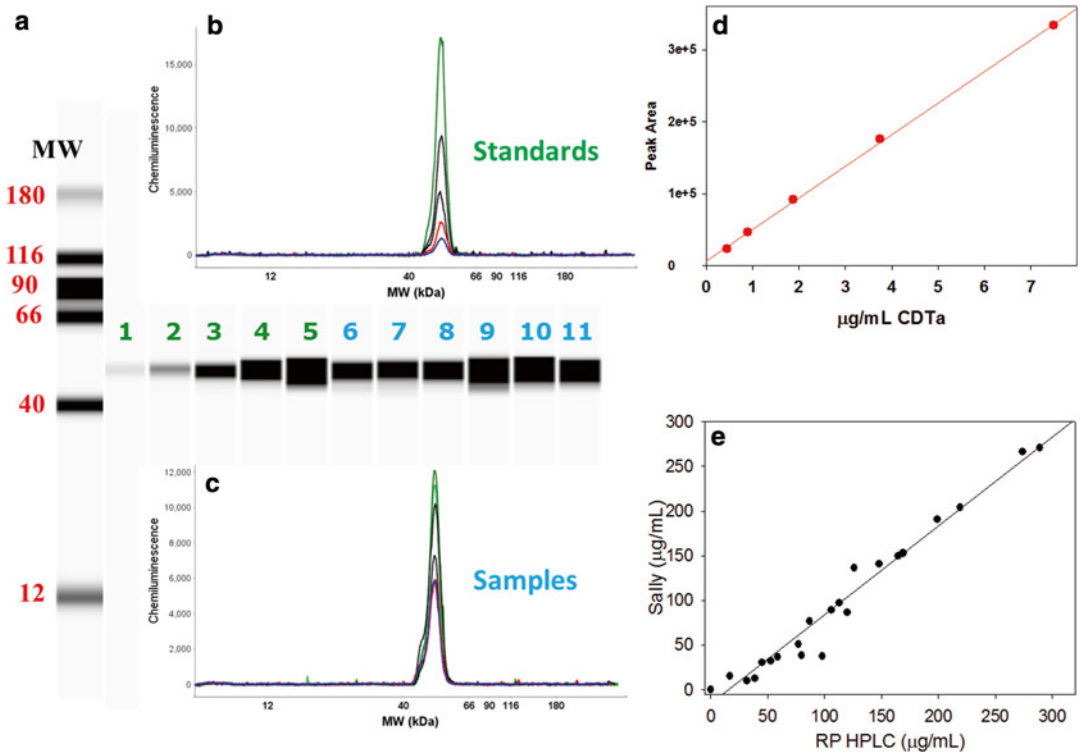

Fig. 4 An example of CDTa titer screening in various cell culture conditions and using Simple Western. A five-point standard curve was run together with many cell culture samples on a 96-capillary Sally. (**a**) Gel image of standard curve (lane 1–5) and samples (lane 6–11); (**b**) Electropherograms of standard curve in (**a**); (**c**) Electropherograms of samples in (**a**); (**d**) Linearity of standard curve from 0.45 to 7.5 µg/mL with $R^2 > 0.990$; (**e**) CDTa titer correlation curve between Sally and the traditional RP HPLC method [16, 24] with $R^2 > 0.967$

1. Prepare sample plate by adding 15 µL of prepared samples, primary antibody, secondary antibody, and antibody diluent according to individual instrument user's manual.
2. Prepare Simple Western instrument parameters according to system user's manual (*see* **Note 23**).
3. Perform self-test check (*see* **Note 22**).
4. Click run.
5. Analyze data using Compass software (*see* **Note 24**).
6. If results are not satisfactory, then perform CE and western optimizations.
7. Optimize standard CE parameters (*see* **Note 28**).
8. Optimize standard western blot parameters if necessary (*see* **Note 29**).

The first example where the charge-based western shows utility is in analyzing heat-stressed stability samples of final container drug product of tetravalent *C. difficile* vaccine. Figure 6 illustrates

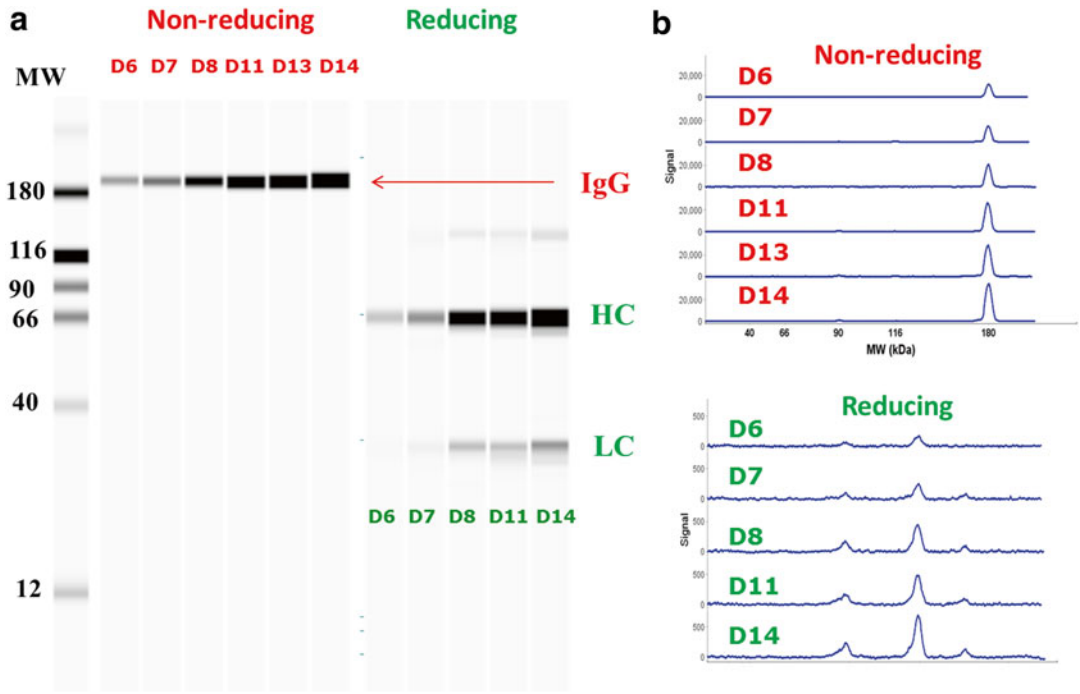

Fig. 5 (**a**) Virtual gel image of therapeutic mAb production in CHO cell culture from day 6 to 14 as monitored under both nonreducing and reducing conditions with Simple Western. A 20× dilution of human anti-(HC + LC)-HRP obtained from Jackson Immuno Research was used. Iodoacetamide was added in the nonreducing condition. An increase of IgG production is observed from day 6 to 14 without any clipping or misfolding between HL-LC and HC-HC observed. The method can be used as an alternative to both Protein A HPLC IgG titer assay and IgG product quality (see also Fig. 7b for charge-based western data); (**b**) Electropherograms of (**a**)

the capillary IEF western for TcdA (pI = 5.9), CDTa (pI = 8.7), and proCDTb (pI = 4.5) final drug product before (blue) after (red) an accelerated heat stress at 37 °C study. We are not able to obtain a reproducible IEF profile for TcdB antigen. There is a decrease in intensity for TcdA after heat stress, while there is a slight increase in acidic variant peaks for proCDTb. For CDTa two peaks are present of which the acidic peak increases when heat-stressed. For CDTa and proCDTb, the size-based western shows one peak with no change after heat stress while the charge-based western detected charge heterogeneity, with the acidic peak increasing upon stress, demonstrating how both techniques are complementary. Understanding acidic variants of proteins and the impact of heat stress on these variants is important and often this can be caused by deamidation. It has been demonstrated for some mAb products that their acidic variants are important for biological activity [25, 26]; therefore the acidic variant in these cases becomes a critical quality attribute. The analysis of these acidic variants is very labor intensive involving multiple analytical methods including HPLC, MS, cIEF, and ELISA [27]. The native charge-based western can be used as a fast surrogate biological activity assay for acidic

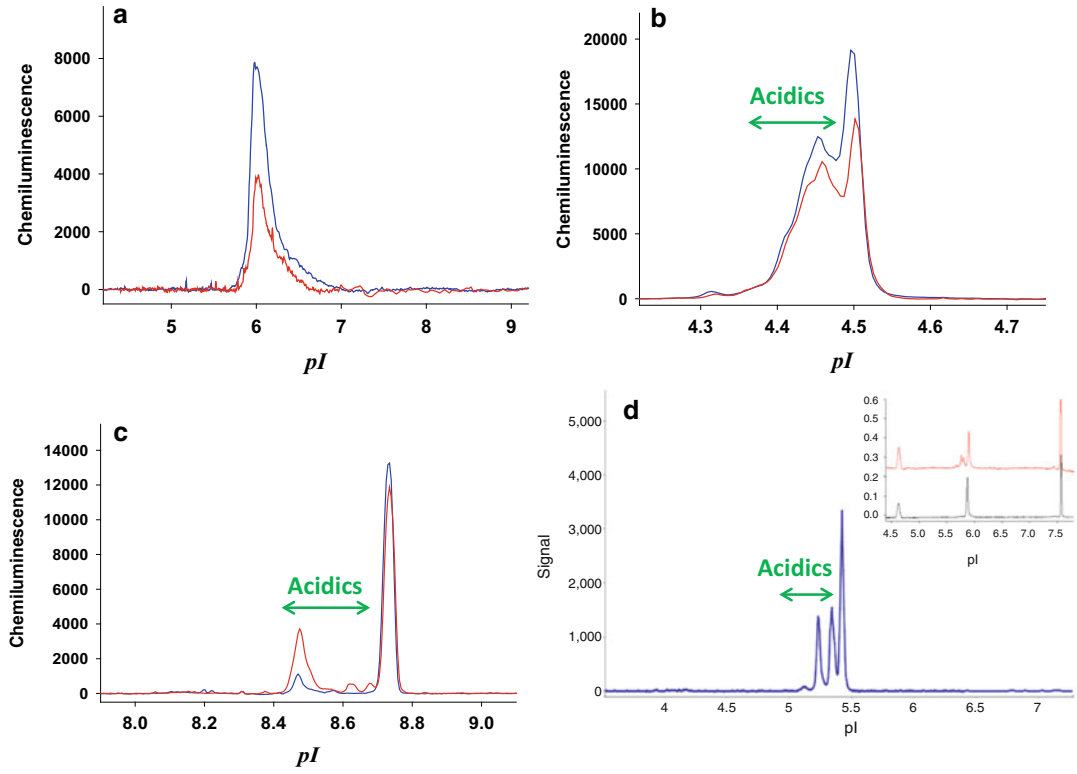

Fig. 6 Stability indicating assay for tetravalent *C. difficile* vaccine drug product evaluated using charge-based western at −70 °C (*blue*) and 14 days at 37 °C (*red*). (**a**) TcdA decreases in intensity at 37 °C. Ampholytes 3–10 (1000 μL) range was used with pI ladder 3 (60 μL); (**b**) ProCDTb shows a slight increase in acidic variants. Note that a slightly lower intensity observed for proCdtB peak at 37 °C was attributed to sample preparation. Ampholytes 4–7 (1000 μL) range was used with pI ladder 3 (60 μL); (**c**) CDTa shows two charge variants and the acidic portion has increased while the main peak decreases. Ampholyte-free solution (888 μL) was mixed with pharmalytes 8–10.5 (88 μL) with pI ladder 3 (60 μL); (**d**) Charge profile of CRM197 stressed material with final concentration ~5 μg/mL. The ampholyte 3–10 (700 μL) range was used with pI ladder 3 (50 μL). Four peaks are observed by simple western after 3 months at 25 °C indicating that all three acidic variants are immunoreactive and hence biologically active. The inset shows an imaged cIEF profile of CRM197 control (*black*) and stressed 3 months 25 °C (*red*) material which also showed four variants (one main and three acidic peaks) after stress [23]. Peggy instrument default focusing parameters were employed for all charge-based western

variants as demonstrated in Fig. 6d. The imaged capillary IEF of CRM197 is illustrated on the inset of Fig. 6d showing the IEF profile of CRM197 (pI = 5.8) and that acidic variants increase after 3 months at 25 °C [23]. When this CRM197 is probed by its biologically relevant mAb in a charge-based simple western, the three acidic variants are detected indicating that all are biologically active.

The second example for charge-based western used is during biosimilar development of the heavily sialylated Fc-fusion protein, Enbrel. This technique is used to quickly screen cell culture samples from various clones for production of Enbrel produced in a *Pichia pastoris* expression system and compared to the

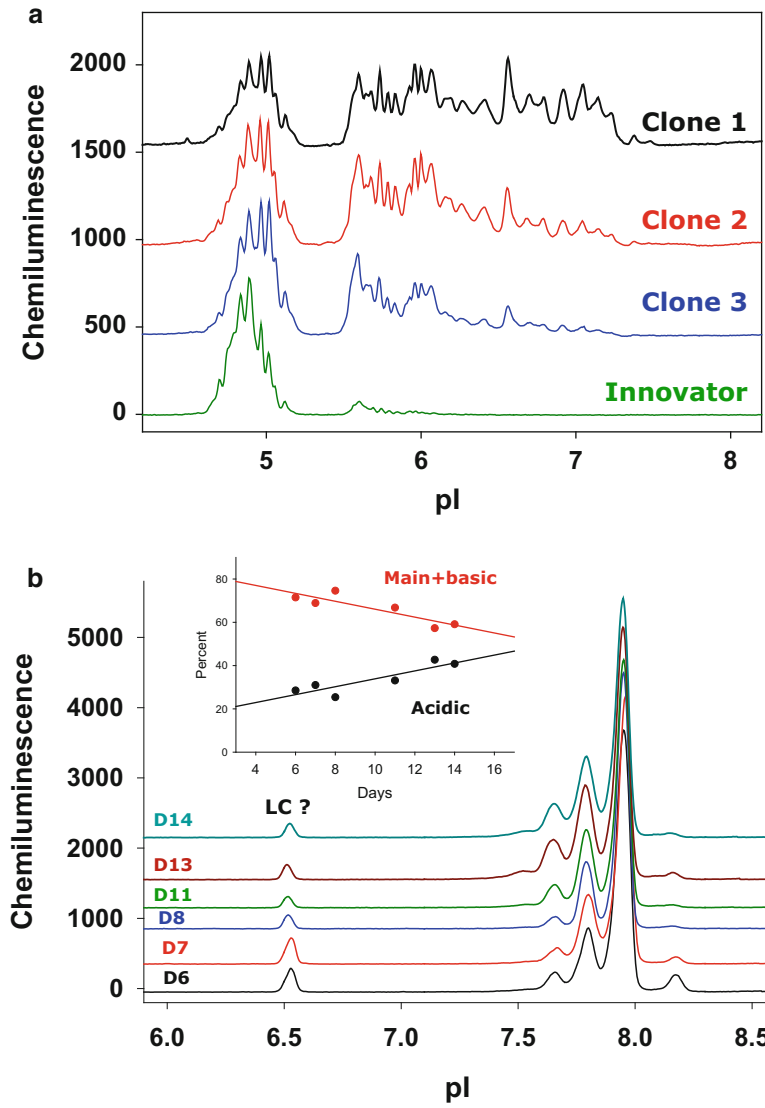

Fig. 7 (**a**) The charge-based western (Peggy) was used to analyze samples as part of biosimilar development of Enbrel produced from *Pichia pastoris*. Charge profiles are shown for three clones compared to the innovator. All sample final concentrations were 0.5 μg/mL. Ampholyte 4–9 (1050 μL) range was used with pI ladder 1 (23 μL). (**b**) The same mAb production samples as in Fig. 5 were monitored for charge heterogeneity. Electropherograms from day 6 to 14 are shown and the percent acidic variants increase during production (see inset graph). The extra peak at pI = 6.5 is likely LC since there is always more LC than HC during cell culture production (note that this was not observed in the size-based western in Fig. 5 and was likely caused by sensitivity, since it is known that in IEF the sample is concentrated at least 10× or higher after focusing). The final mAb concentration was 2 μg/mL and the ampholyte 3–10 (500 μL) range was used with pI ladder 1 (20 μL). Peggy instrument default focusing parameters were employed for all charge-based western

innovator. The charge distribution profiles are shown in Fig. 7a. Although Enbrel from clone 3 looks slightly better than clone 2 or 1 in terms of its charge variant distribution, it is still far cry from its innovator profile. The method has helped the evaluation of various clones early in cell culture to match the innovator charge variant profile. This demonstrates the use of this technique as a quick screen of low volume, upstream, cell culture samples (prior to purification) for development of a biosimilar therapeutic protein. The method can be applied to other heavily sialylated proteins such as erythropoietin (EPO).

The third example where the charge-based western has shown usefulness is for monitoring charge heterogeneity in therapeutic mAb production during cell culture fermentation. Figure 7b illustrates the capillary IEF western electropherograms from day 6 to day 14 of mAb production. The inset graph demonstrates that the acidic variants increase during mAb production. This early information could become very important if the acidic variants are critical and the quantity of these variants can be controlled by varying the harvest time. Again this demonstrates that the charge-based Simple Western is a complementary tool to the size-based western as no size heterogeneity was detected during fermentation.

4 Notes

1. ProteinSimple has come out with five different instruments for the size-based Simple Western in the last 3 years. All instruments use the same technology but vary by modes of separation, number of capillaries, and sensitivity. The first generation is called Simon which runs 12 capillaries and is being discontinued. The first 96-capillary version for size is called Sally, and was followed by Peggy, which can run both size- and charge-based capillary westerns. Later they launched Sally Sue and Peggy Sue which are more sensitive versions of Sally and Peggy. They are planning to eventually phase out Sally and Peggy, but the new reagents for Sally Sue and Peggy Sue can be used for Sally and Peggy, which will increase sensitivity without the need for upgrading instruments. Similarly, reagents from Sally and Peggy can be used on the new Sally Sue and Peggy Sue but sensitivity will decrease compared with using the Sue platform reagents. Finally, Wes is the most recently introduced instrument and is a 25 capillary version of Simple Western. The reagents for Wes are identical to Sally Sue or Peggy Sue, but most reagents for Wes are preplated and one only needs to prepare and add samples, luminol S/peroxide mixture, antibody diluent, primary and secondary antibodies, and wash buffer. The benefit of Wes is that sensitivity is as high as Sally Sue and Peggy Sue and it runs much faster than the other four instruments.

2. The total volume of a 5 cm capillary is approximately 400 nL. When the default vacuum injection is used for each instrument, the total volume taken up in the capillary is about 40 nL for the size-based western. In the charge-based western, the whole capillary is filled with sample (400 nL), then focused by a pH gradient after applying voltage. It is not recommended to use size capillary for charge or vice versa.
3. Although the SDS-containing separation matrix and stacking matrix are labeled differently for Sally/Peggy and Sally Sue/Peggy Sue, both can be used interchangeably without noticing any differences. The running buffer is the same for all instruments. These three reagents are already in the prefilled plate for Wes.
4. The matrix removal buffer and concentrated wash buffer are only for Sally/Peggy. For Sally Sue/Peggy Sue, matrix removal becomes lower running buffer and running buffer becomes upper running buffer, while wash buffer is the same as in Sally/Peggy. This change was recently implemented by Protein Simple. There is no matrix removal buffer in Wes and concentrated wash buffer can be used in Wes by diluting it three times.
5. Luminol S, peroxide, sample buffer 10×, and Streptavidin-HRP are the same for all size-based instruments. Sample buffer 10× can be substituted by preparing 100 mM Tris, 10% SDS pH 7.5. Streptavidin-HRP can be purchased from Jackson Immuno Research and when diluted three times, it will give about a similar intensity as Streptavidin-HRP from ProteinSimple.
6. Although both goat anti-rabbit and goat anti-mouse mAbs are different for Sally/Peggy and Sally Sue/Peggy Sue/Wes instruments, the ones that are designated for Sally Sue/Peggy Sue can be used for Sally/Peggy and the sensitivity will increase significantly. Different antibodies such as anti-donkey-HRP, anti-sheep-HRP, anti-monkey-HRP, and anti-human-HRP can be purchased from Jackson Immuno research and work well with some optimization such as dilution and incubation time.
7. ProteinSimple came out with three different types of antibody diluents (proteins-containing blocking reagent similar to milk or casein for manual western). These three antibody diluents (antibody diluent, antibody diluent plus, and antibody diluent II) can be used on all instruments but sensitivity may vary.
8. Fluorescent protein markers for size-based western contain three different proteins labeled covalently with a fluorescent dye that function mainly for alignment (registration) using the 1 kDa protein and coarse molecular weight standards (if these standards are used instead of the biotinylated MW markers, MW determination in the samples will be inaccurate) in Sally/Peggy and Sally Sue/Peggy Sue. The first generation fluorescent standard markers contain 1, 29, and 180 kDa, while the

second generation has 1, 29, and 230 kDa. Both generations will work on all size-based instruments. It is also very important to perform a negative control experiment of your primary antibody against these fluorescent protein markers for possible cross reactivity. Cross reactivity has been observed for various antibodies especially with the high MW protein markers, 180 and 230 kDa.

9. A large batch of these MW markers can be prepared, and they can be aliquoted at 8 μL each for single use and stored at −70 °C for up to 1 year. Custom biotinylated MW markers can be made by labeling the proteins of interest with biotin molecule; however, any residual biotin needs to be removed because biotin will be separated and detected on this Simple Western system. Though surprising, we speculate the separation mechanism is not by MW but rather pseudo-MEKC [21].
10. The master mix (2×) reducing solution preparation is not the same as ProteinSimple's recommendation because we found that increasing the amount of DTT and SDS in final sample buffer yields better reproducibility especially for crude lysate samples or membrane containing proteins. Increasing DTT helps in removing higher order disulfide-linked oligomers sometimes observed for membrane proteins which are very critical for accurate quantitation. Master mix (2×) nonreducing solution can be prepared similarly by replacing the 200 mM DTT with 200 mM Iodoacetamide (this minimizes the disulfide shuffling during sample denaturation and heating).
11. The sample heating condition should be optimized for each protein. For example, mAbs are well known to degrade, forming a thioether bond between LC-HC, and increase disulfide shuffling if it is heated above 70 °C [6, 9, 28]. However, some membrane proteins containing coiled-coil motif anchored and β-strands or β-barrel structures need at least 95 °C or above to be efficiently denatured and bind SDS [29].
12. The charge-based western was actually the first instrument that came to the market in 2006 and it was called the FireFly™ 3000 blotless immunoassay which then later became the CB1000 and finally was called the NanoPro 1000. This NanoPro 1000 could only do the IEF-based western, but later it got upgraded to Peggy and Peggy Sue which can run both charge- and size-based westerns. Unlike size-based western, there is no difference in terms of performance in charge-based westerns between Peggy and Peggy Sue.
13. Do not mix luminol/peroxide reagents for charge and size, as they are not identical.
14. Bicine chaps is used for dilution but primarily for crude cell lysate to help cell lysis. However, for native condition, it is preferred to remove bicine chaps if possible.

15. Each pI standard set consists of five different fluorescently labeled pI markers (these are very similar to the fluorescently labeled protein standards in size-based western). The pI ladder 1 has pI 4.0, 4.9, 6.0, 6.4, and 7.3; while pI ladder 3 contains pI 4.9, 6.0, 6.4, 7.0, and 7.3.
16. There are individual fluorescently labeled pI markers that are added together with the pI ladder when very basic or acidic proteins are measured since all pI ladder sets do not contain very high pI markers.
17. Premix ampholyte-free solution is used by mixing it with other commercial ampholytes such as pharmalytes, servalytes, or biolytes. ProteinSimple also provides ready to use premix with ampholyte added at varying ranges.
18. pI ladder and ampholyte premix are chosen as appropriate to bracket the sample of interest. Combinations of these and conditions may be optimized for the given application but a 20:1 (premix:pI ladder) ratio is a good starting point.
19. Antibody dilution must be optimized for each application. A 1:100 dilution for primary and secondary antibody is a good starting point but a range of dilutions should be tested (*see* **Note 26**).
20. The linear range of the assay needs to be determined for each analyte separately and will be dependent on sample type, antibodies, and other factors. For a purified protein or vaccine typically 1 μg/mL of protein was within the linear range for most applications so this may be a good starting place. For crude upstream samples, the dilution will vary depending on the amount of antigen present. However, some dilution in detergent or lysis buffer may be needed to solubilize cells and extract proteins of interest. Bicine chaps is a standard diluent but ProteinSimple has other recommended dilution and lysis buffers that can be used.
21. It is important to use pipettes suitable for pipetting very viscous solutions accurately. In addition it is important to carefully add the sample solution to the vial so as to avoid the introduction of air bubbles into the solution. Air bubbles in the sample solution injected into the separation capillary will result in noise spikes in the electropherogram. Lastly, mixing by vortexing is critical as the viscous premix and sample must be thoroughly mixed for accurate quantitation.
22. It is important to perform self-test under the instrument tab on the main menu especially when the instrument has been idle for a long period of time.
23. Since Simple Western is a combination between CE and western blot, both portions of the assay should be optimized for each protein. Default conditions from the manufacturer can

be used but these conditions may be suboptimum for the proteins of interest.

24. Although the instrument software Compass is adequate in most cases, it is still highly recommended to transfer data and analyze using a standard HPLC software package such as Empower.
25. Standard CE optimization steps can be applied here such as varying stacking and sample injection times (sample volume) to obtain resolution and sensitivity. Resolution is usually the least important in a western blot since in most cases we are only interested in one protein, and hence improving sensitivity is more critical. A field amplified stacking, as is in the Simple Western system, can yield a tenfold or more improvement in sensitivity by varying stacking times to up 20 s and by longer sample injection times to up 15–20 s. When the stacking time is increased significantly, the separation time may also need to be reduced or the 1 kDa marker could come off from the capillary and the ability to align the sample will be lost. If greater resolution between two proteins is needed, then a higher stacking to sample loading ratio is preferred especially in the region of the higher MW proteins. Other CE parameters that can be optimized are separation time and voltage to reduce the capillary gel electrophoresis separation time. Since resolution is not typically important in western blot, separation time can be decreased by either increasing the voltage or reducing separation run time. When voltage is increased, the current needs to be monitored and maintained <150 μA for Sally/Peggy and Sally Sue/Peggy Sue, and <400 μA for Wes to reduce Joule heating.
26. Western blot optimization is very similar to a manual western with respect to optimizing primary antibody dilution and primary and secondary antibody incubation times. In general, decreasing antibody incubation times from the default will reduce signal intensity and background; however if sensitivity is not required reducing antibody incubation times as much as 50% can be done. Primary antibody dilution depends significantly on how well it binds its target protein. Dilution can be varied between 10× and 1000× dilution from a standard 1 mg/mL concentration of mAb. Generally lower mAb dilution gives both higher signal and background similar to longer antibody incubation times; therefore the balance between the signal to noise ratio needs to be considered when adjusting these parameters. Polyclonal antibodies produced in rabbits usually provide higher signal than monoclonal produced in mice; however, polyclonals can have more cross reactivity with other proteins especially if the source of the polyclonal is from a commercial vendor. If a polyclonal reagent is generated by immunizing rabbits using antigens with >90% purity, the poly-

clonal serum reagent usually has low/zero cross reactivity with other proteins and a 1000×–10,000× pAb dilution can be achieved. Two types of secondary antibody are available from ProteinSimple, anti-rabbit and anti-mouse (rarely dilution optimizations are needed). Finally, another parameter that can be changed to decrease the total run time is the chemiluminescence detection times. The instrument default usually provides 6 different detection times varying from 5 to 960 s. In most cases longer detection time such as 960, 480, and 240 s are not needed especially when the assay produces strong signal. If these portions of the default method detection times are eliminated, it reduces the total run time by almost 30 min for each cycle. Overall, with these adjustments, the total run time can be reduced from 22 h for all 96 capillaries to about 16–18 h, while for Wes the total run time can be decreased from 3 to 2 h.

27. The TcdA (MW ~308 kDa) and TcdB (MW ~270 kDa) bands appear above 180 kDa MW marker because these MWs are beyond the range of the separation gel matrix of 12–180 kDa (stacking and sample injection times were adjusted accordingly; see figure legend). The stock separation gel matrix could be diluted to 80% with water which might give better separation, but ProteinSimple has just recently came out with a separation matrix gel for high MW protein ranging from 66 to 440 kDa.
28. In general, method development of capillary IEF is much more difficult than CE-SDS gel (CGE) methods for protein because protein isoelectric focusing depends on many parameters such as ampholyte mixture concentration, low ionic buffer concentration (<20 mM), and focusing time (5–30 min). Additionally, a potential protein precipitation exists at its pI which may cause a requirement for additives such as a non-ionic surfactant, urea, or glycerol to prevent it. Furthermore, when signal is not detected in charge-based western, it is sometimes difficult to troubleshoot the root cause (i.e., protein is not focused or primary antibody is not good).
29. Instrument running conditions may be optimized outside of the vendor recommended defaults. For instance, the antibody incubation time, exposure times, wash times, and other conditions may be changed to optimize each assay similar to the size-based western (*see* **Note 26**).

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References

1. Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to NC sheets: procedure and applications. Proc Natl Acad Sci U S A 76:4350–4354
2. Renart J, Reiser J, Stark GR (1979) Transfer of proteins from gels to diazobenzyloxymethyl paper and detection with anti-sera: a method for studying antibody specificity and antigen structure. Proc Natl Acad Sci U S A 76:3116–3120
3. Kurien BT, Scofield RH (2006) Western blotting. Methods 38:283–293
4. Ghosh R, Gilda JE, Gomez AV (2014) The necessity of and strategies for improving confidence in the accuracy of Western Blots. Expert Rev Prot 11:549–560
5. Koller A, Wätzig H (2005) Precision and variance components in quantitative gel electrophoresis. Electrophoresis 26:2470–2475
6. Hunt G, Nashabeh W (1999) Capillary electrophoresis sodium dodecyl sulfate nongel sieving analysis of a therapeutic recombinant monoclonal antibody: a biotechnology perspective. Anal Chem 71:2390–2397
7. Ma S, Nashabeh W (2001) Analysis of protein therapeutics by capillary electrophoresis. Chromatographia 53:S75–S89
8. Rustandi RR, Washabaugh MW, Wang Y (2008) Application of CE SDS gel in development of biopharmaceutical antibody-based products. Electrophoresis 29:3612–3620
9. Rustandi RR, Wang Y (2011) Use of CE-SDS gel for characterization of monoclonal antibody hinge region clipping due to copper and high pH stress. Electrophoresis 32: 3078–3084
10. Rustandi RR, Anderson CL, Hamm M (2013) Application of capillary electrophoresis in glycoprotein analysis. In: Beck A (ed) Glycosylation engineering of biopharmaceuticals methods and protocols, vol 988. Humana Press, Totowa, NJ, pp 181–197
11. Wu J, Wu XZ, Huang T, Pawliszyn J (2004) Analysis of proteins by CE, CIEF and microfluidic devices with whole-column-imaging detection. In: Strege MA, Lagu AL (eds) Methods in molecular biology: capillary electrophoresis of proteins and peptides, vol 276. Humana Press, Totowa, NJ, pp 229–252
12. Anderson C, Wang Y, Rustandi RR (2012) Applications of imaged capillary isoelectric focusing technique in development of biopharmaceutical glycoprotein-based product. Electrophoresis 33:1538–1544
13. Rustandi RR, Wang F, Hamm C, Cuciniello JJ, Marley ML (2014) Development of imaged capillary isoelectric focusing method and capillary zone electrophoresis in hepatitis B vaccine RECOMBIVAX HB®. Electrophoresis 35: 1072–1078
14. O'Neill RA, Bhamidipati A, Bi X, Deb-Basu D, Cahill L, Ferrante J, Gentalen E, Glazer M, Gossett J, Hacker K, Kirby C, Knittle J, Loder R, Mastroieni C, MacLaren M, Mills T, Nguyen U, Parker N, Rice A, Roach D, Suich D, Voehringer D, Voss K, Yang J, Yang T, Vander Horn PB (2006) Isoelectric focusing technology quantifies protein signaling in 25 cells. Proc Natl Acad Sci U S A 103: 16153–16158
15. Nguyen U, Squaglia N, Boge A, Fung PA (2011) The simple Western™: a gel-free, blot-free, hands-free Western blotting reinvention. Nat Methods 8:v–vi
16. Rustandi RR, Loughney JW, Hamm M, Hamm C, Lancaster C, Mach A, Ha S (2012) Qualitative and quantitative evaluation of Simon™, a new CE-based automated western blot system as applied to vaccine development. Electrophoresis 33:2790–2797
17. Chen J-Q, Heldman MR, Herrmann MA, Kedei N, Woo W, Blumberg PM, Goldsmith PK (2013) Absolute quantitation of endogenous proteins with precision and accuracy using a capillary Western system. Anal Biochem 442:97–103
18. Liu S, Sardi S, Sonom B, Zocco D, McSweeney R, Fraser A, Halleck A, Li H, Smejkal G, Munevar S, Jin JG, Kawai T, Ghiran I, McGrath JP, Whitman M, Ng S, Kuo WP (2013) Application of a novel nanovolume capillary electrophoresis based protein analysis system in personalized and translational medicine research. J Bioanal Biomed S3:1–10
19. Loughney JW, Lancaster C, Ha S, Rustandi RR (2014) Residual bovine serum albumin (BSA) quantitation in vaccines using automated capillary Western technology. Anal Biochem 461:49–56

20. Hamm M, Ha S, Rustandi RR (2015) Automated capillary Western dot blot method for the identity of 15-valent pneumococcal conjugate vaccine. Anal Biochem 478: 33–39
21. Rustandi RR, Hamm M, Loughney JW, Ha S (2015) Detection of ADP-ribosylation in PARP-1 and bacterial toxins using a capillary-based western system. Electrophoresis 36: 2798-2804. http://onlinelibrary.wiley.com/doi/10.1002/elps.201500173/pdf
22. Wang B, Wang S, Rustandi RR, Wang F, Mensch CD, Hong L, Kristopeit A, Secore S, Dornadula D, Kanavage T, Heinrichs JH, Mach H, Blue JT, Thiriot DS (2015) Detecting and preventing reversion to toxicity for a formaldehyde-treated *C. difficile* toxin B mutant. Vaccine 33:252–259
23. Rustandi RR, Peklansky B, Anderson CL (2014) Use of imaged capillary isoelectric focusing technique in development of diphtheria toxin mutant CRM197. Electrophoresis 35:1065–1071
24. Lancaster C, Pannizzo P, Ha S, Rustandi RR (2014) A reserve phase HPLC mass assay for *C. difficile* binary toxin vaccine. HPLC 2014 Poster P-W-2005, New Orleans
25. Harris RJ, Kabakoff B, Macchi FD, Shen FJ, Kwong M, Andya JA, Shire SJ, Bjork N, Totpal K, Chen AB (2001) J Chromatogr A 752:233–245
26. Vlasak J, Bussat MC, Wang S, Wagner-Rousset E, Schaefer M, Klinguer-Hamour C, Kirchmeier M, Corvaïa N, Ionescu R, Beck A (2009) Identification and characterization of asparagine deamidation in the light chain CDR1 of a humanized IgG1 antibody. Anal Biochem 392:145–154
27. Wang F, Peklansky B, Anderson C, Wang Y, Rustandi RR (2012) Improved ion-exchanged HPLC method in mAb using pH gradient and its comparison with cIEF. J Liq Chrom Rel Tech 35:1259–1269
28. Cohen SL, Price C, Vlasak J (2007) β-Elimination and peptide bond hydrolysis: two distinct mechanisms of human IgG1 hinge fragmentation upon storage. J Chem Am Soc 129:6976–6977
29. Commanducci M, Bambini S, Brunelli B, Adu-Bobie J, Arico B, Capecchi B, Giuliani MM, Masignani V, Santini L, Savino S, Granoff DM, Caugant DA, Pizza M, Rappuoli R, Mora M (2002) NadA, a novel vaccine candidate of *Neisseria meningitidis*. J Exp Med 195: 1445–1454

Chapter 17

Affinity Capillary Electrophoresis Applied to Investigation of Valinomycin Complexes with Ammonium and Alkali Metal Ions

Sille Štěpánová and Václav Kašička

Abstract

This chapter deals with the application of affinity capillary electrophoresis (ACE) to investigation of noncovalent interactions (complexes) of valinomycin, a macrocyclic dodecadepsipeptide antibiotic ionophore, with ammonium and alkali metal ions (lithium, sodium, potassium, rubidium, and cesium). The strength of these interactions was characterized by the apparent binding (stability, association) constants (K_b) of the above valinomycin complexes using the mobility shift assay mode of ACE. The study involved measurements of effective electrophoretic mobility of valinomycin at variable concentrations of ammonium or alkali metal ions in the background electrolyte (BGE). The effective electrophoretic mobilities of valinomycin measured at ambient temperature and variable ionic strength were first corrected to the reference temperature 25 °C and constant ionic strength (10 or 25 mM). Then, from the dependence of the corrected valinomycin effective mobility on the ammonium or alkali metal ion concentration in the BGE, the apparent binding constants of the valinomycin–ammonium or valinomycin–alkali metal ion complexes were determined using a nonlinear regression analysis. Logarithmic form of the binding constants (log K_b) were found to be in the range of 1.50–4.63, decreasing in the order $Rb^+ > K^+ > Cs^+ >> Na^+ > NH_4^+ \sim Li^+$.

Key words Valinomycin, Valinomycin complexes, Affinity capillary electrophoresis, Binding constant, Stability constant, Peptide complexes, Non-covalent interactions

1 Introduction

The transport of ions in and out of living cells is vital to many biological processes, such as conservation and transfer of genetic information, signal transduction, and regulation of enzymatic activity. Ionophores transporting ions across natural and artificial membranes exhibit antibiotic, apoptotic, insecticide, and other relevant properties and therefore attract a broad scientific interest [1–4]. Antibiotic valinomycin (Val) has been one of the first compounds to be recognized as ionophore [5, 6]. The specific function of an ionophore depends on its selectivity and kinetics of ion capture, transport, and release. Val selectively transports

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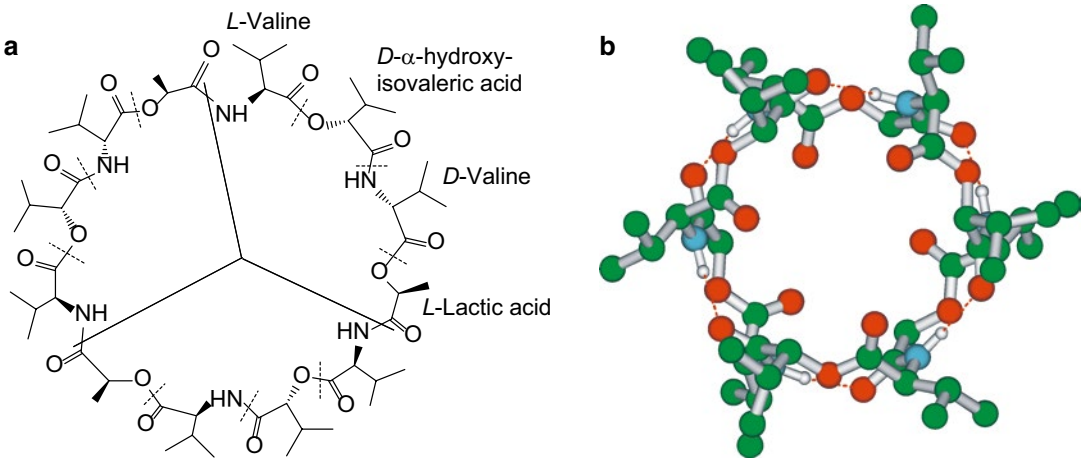

Fig. 1 (**a**) Structural formula of valinomycin. (**b**) Projection of valinomycin structure calculated by density functional theory (hydrogen atoms omitted for clarity except six hydrogens taking part in six internal hydrogen bonds between the nearest depsipeptide units). Adapted with permission from [10]

potassium ions across cell membrane bilayers. Val is a macrocyclic dodecadepsipeptide consisting of three identical fragments (L-valine–D-α-hydroxy-isovaleric acid–D-valine–L-lactic acid); its 36-membered ring contains alternating six amide and six ester bonds; *see* Fig. 1a. The valinomycin structure, in the absence of bound cations, depends on the polarity of the solvent. In nonpolar solvents valinomycin adopts a bracelet-like conformation and in polar solvents a propeller-like conformation [7]. However, when bound to potassium or similar size ions, it adopts a configuration in which its amino acid and hydroxy acid side chains always point outward, creating a hydrophobic exterior, while the six carbonyl oxygen atoms from the hydroxy acid residues point inward, forming a cavity for ion complexation [4]; *see* Fig. 1b.

Quantum chemical study has demonstrated that high selectivity of Val for K^+ ion over Na^+ ion is caused by its cavity constraints (rigidity) that physically prevent it from collapsing onto smaller Na^+ ion [1–4]. These constraints are enforced by a combination of intramolecular hydrogen bonds and other structural features including its specific ring size and the spacing between its connected ligands. Besides K^+ and Na^+ ions, Val binds also several other metal and non-metal inorganic and organic cations [1–4]. Strength of Val noncovalent interactions with these ions is characterized by the binding (stability, association) constants of the appropriate Val complexes. The binding constants of Val complexes determined by various methods (UV–Vis spectrophotometry and fluorimetry, potentiometry, and electric conductivity) can be found in a review article [8] and references cited therein. Recently, the valinomycin complexes with ammonium and alkali metal ions (lithium, sodium, potassium, rubidium, and cesium)

were investigated by affinity capillary electrophoresis (ACE) and density functional theory (DFT) in our laboratory [9–13]. Mobility shift mode of ACE [14–17] was employed for determination of apparent binding constants of Val complexes in methanol, and by means of DFT calculations, the structural details of Val complexes, such as position of the ion in the cavity of Val molecule and the interatomic distances within the complex, were described. Below, the practical aspects of the ACE investigations of Val complexes are summarized and the obtained values of apparent binding constants of these complexes are presented and compared with literature values determined by other methods.

2 Materials and Instrumentation

2.1 Valinomycin Complexes with K^+, Rb^+, Cs^+, and NH_4^+ Ions

2.1.1 Stock Solutions

1. 200 mM Acetic acid: To prepare 100 mL: 1.2010 g of acetic acid was dissolved in 95 mL methanol. Methanol was added to reach a final volume of 100 mL using a volumetric flask.
2. 200 mM Tris(hydroxymethyl)aminomethane (Tris): To prepare 100 mL: 2.4228 g of Tris was dissolved in 95 mL methanol. Methanol was added to reach a final volume of 100 mL using a volumetric flask.
3. 100 mM Tris, 50 mM acetic acid: To prepare 100 mL: 50 mL of 200 mM Tris, 25 mL of 200 mM acetic acid, and methanol to reach the final volume of 100 mL using a volumetric flask. The pH_{MeOH} values of the BGE according to the apparent, and conventional pH scale, described by Porras et al. [18] were 8.51 and 10.18, respectively, using the pK_a values for acetic acid and Tris in methanol to be equal to 9.70 [18] and 10.18 [19], respectively.
4. 10 mM of KCl: To prepare 10 mL: 0.0075 g of KCl was dissolved in 8 mL methanol. Methanol was added to reach a final volume of 10 mL using a volumetric flask.
5. 10 mM RbCl: To prepare 10 mL: 0.0121 g of RbCl was dissolved in 8 mL methanol. Methanol was added to reach a final volume of 10 mL using a volumetric flask.
6. 10 mM CsCl: To prepare 10 mL: 0.0168 g of CsCl was dissolved in 8 mL of methanol. Methanol was added to reach a final volume of 10 mL using a volumetric flask.
7. 100 mM NH_4Cl: To prepare 25 mL: 0.1336 g of NH_4Cl was dissolved in 20 mL of methanol. Methanol was added to reach a final volume of 25 mL using a volumetric flask.
8. 1 mM Val: To prepare 10 mL: 0.0111 g of Val was dissolved in 8 mL of 100 mM Tris, 50 mM acetic acid BGE (without KCl/RbCl/CsCl/NH_4Cl). The same solution was added to reach a final volume of 10 mL using a volumetric flask.

9. 10 mM Dimethyl sulfoxide (DMSO): To prepare 10 mL: 7.1 µL of DMSO was dissolved in 8 mL of 100 mM Tris, 50 mM acetic acid BGE (without KCl/RbCl/CsCl/NH_4Cl). The same solution was added to reach a final volume of 10 mL using a volumetric flask.

2.1.2 Working Solutions

1. BGEs: 100 mM Tris, 50 mM acetic acid containing 0–1 mM KCl/RbCl/CsCl or 0–40 mM of NH_4Cl. To prepare 10 mL: 0–1 mL of 10 mM KCl/RbCl/CsCl or 0–4 mL of 100 mM NH_4Cl and 100 mM Tris-50 mM acetic acid BGE to reach the final volume of 10 mL using a volumetric flask.
2. 45 µM Val: To prepare 5 mL: 225 µL 1 mM Val and 100 mM Tris-50 mM acetic acid BGE to reach the final volume of 5 mL using a volumetric flask.
3. 1 mM Dimethyl sulfoxide: To prepare 10 ml: 1 mL of 10 mM DMSO and 100 mM Tris-50 mM acetic acid BGE to reach the final volume of 10 mL using a volumetric flask.

2.2 Valinomycin Complex with Na^+ Ion

2.2.1 Stock Solutions

1. 20 mM Chloroacetic acid, 10 mM Tris: To prepare 100 mL: 10 mL of 200 mM chloroacetic acid, 10 mL of 100 mM Tris, and methanol to reach the final volume of 100 mL using a volumetric flask. The pH_{MeOH} of the BGE according to the conventional pH scale was 7.8 (the pK_a value of chloroacetic acid in methanol at 25 °C is 7.8) [18].
2. 100 mM NaCl: To prepare 25 mL: 0.1461 g of NaCl was dissolved in 20 mL methanol. Methanol was added to reach a final volume of 25 mL using a volumetric flask.
3. 10 mM Mesityl oxide: To prepare 10 mL: 12.7 µL of mesityl oxide was dissolved in 8 mL of methanol. Methanol was added to reach a final volume of 10 mL using a volumetric flask.

2.2.2 Working Solutions

1. BGEs: 20 mM Chloroacetic acid, 10 mM Tris, containing 0–40 mM NaCl. To prepare 10 mL: 0–4.00 mL 100 mM NaCl and 20 mM chloroacetic acid–10 mM Tris solution to reach the final volume of 10 mL using a volumetric flask.
2. 180 µM Val: To prepare 5 mL: 900 µL of 1 mM Val and methanol to reach the final volume of 5 mL using a volumetric flask.
3. 2 mM Mesityl oxide: To prepare 10 mL: 2 mL of 10 mM mesityl oxide and methanol to reach a final volume of 10 mL using a volumetric flask.

2.3 Valinomycin Complex with Li^+ Ion

2.3.1 Stock Solutions

1. 200 mM Chloroacetic acid: To prepare 100 mL: dissolve 1.8900 g of chloroacetic acid in 95 mL methanol. Methanol was added to reach a final volume of 100 mL using a volumetric flask.
2. 100 mM Tris: To prepare 100 mL: 1.2114 g of Tris was dissolved in 95 mL methanol. Methanol was added to reach a final volume of 100 mL using a volumetric flask.

3. 100 mM LiCl: To prepare 25 mL: dissolve 0.1060 g of LiCl in 20 mL methanol. Methanol was added to reach a final volume of 25 mL using a volumetric flask.
4. 50 mM Chloroacetic acid, 25 mM Tris: To prepare 100 mL: 25 mL of 200 mM chloroacetic acid, 25 mL of 100 mM Tris, and methanol to reach the final volume of 100 mL using a volumetric flask. The pH_{MeOH} of the BGE according to the conventional pH scale was 7.8.

2.3.2 Working Solutions

1. BGEs: 50 mM Chloroacetic acid, 25 mM Tris containing 0–40 mM LiCl. To prepare 10 mL: 0–4.00 mL of 100 mM LiCl and 50 mM chloroacetic acid–25 mM Tris BGE was added to reach the final volume of 10 mL using a volumetric flask.
2. 360 μM Val: To prepare 5 mL: 1800 μL of 1 mM Val and methanol to reach the final volume of 5 mL using a volumetric flask.
3. 4 mM Mesityl oxide: To prepare 10 mL: 4 mL of 10 mM mesityl oxide and methanol to reach a final volume of 10 mL using a volumetric flask.

2.4 Instrumentation

A homemade CE apparatus [20] equipped with a UV photometric detector monitoring absorbance at 206 nm was used for the ACE experiments. Separations were performed in the internally uncoated fused silica capillary with outer polyimide coating (Polymicro Technologies, Phoenix, AZ, USA), total/effective length 300/190 mm, and id/od 50/375 μm. Chromatography station Clarity (DataApex, Prague, The Czech Republic) was used for data acquisition and evaluation, and program Origin 6.1 (OriginLab, Northampton, MA, USA) was employed for the nonlinear regression analysis.

The apparent pH of the BGE was measured using pH meter CyberScan pH 2100 (Oakton Instruments, Vernon Hills, IL, USA). The electrode was calibrated using aqueous standard pH solutions.

3 Methods

3.1 Preconditioning of the Capillary

1. Before the first use and between the series of analyses in different BGEs, the capillary was conditioned by subsequent rinsing with 0.1 M aqueous sodium hydroxide (10 min), water (10 min), methanol (5 min), and methanolic BGE solution (5 min). All rinsings were performed at 1 bar pressure.
2. Between the runs in the same BGE, the capillary was rinsed for 1 min at pressure 1 bar with methanol and 2 min at pressure 1 bar with the BGE.

3.2 Injection of the Sample Solution

1. Analyte in experiments with K^+, Rb^+, Cs^+, and NH_4^+ ions: 45 μM Valinomycin and 1 mM dimethyl sulfoxide (EOF marker) dissolved in BGE (except for KCl/RbCl/CsCl/NH_4Cl) were introduced into the capillary hydrodynamically, by pneumatically induced pressure (10 mbar) for 30 s.
2. Analyte in experiments with Na^+ ion: 180 μM Valinomycin and 2 mM mesityl oxide (EOF marker), both dissolved in methanol, were consecutively introduced into the capillary by pneumatically induced pressure (10 mbar) for 6 s and 4 s, respectively.
3. Analyte in experiments with Li^+ ion: 360 μM Valinomycin and 4 mM mesityl oxide, both dissolved in methanol, were consecutively introduced into the capillary by pneumatically induced pressure (10 mbar) for 4 s and 3 s, respectively.

3.3 ACE Analyses

After conditioning of the capillary and introduction of the analyte and EOF marker, the CE analysis was performed at a voltage of +12 kV (anode at the injection end). Separations were performed at ambient temperature (24–28 °C), and the measured mobilities were recalculated to reference temperature 25 °C (*see* **Note 1**). As Val formed weak complexes with Li^+, Na^+, and NH_4^+ ions, it was necessary to use higher concentrations of these ions in the BGE and the measured electrophoretic mobilities had to be corrected also to constant ionic strength. The procedure for correction of electrophoretic mobilities to constant ionic strength is described in **Note 2**. The average effective mobilities from three CE experiments were taken for the nonlinear regression analysis of the dependence of effective mobility of Val on the concentration of ammonium and alkali metal ions in the BGE.

3.4 Determination of Binding Constant of Val-Alkali Metal/Ammonium Ion Complexes

1. The ACE method for the determination of the binding constant involved measuring the change in effective electrophoretic mobility of Val as a function of alkali metal or ammonium ion concentration in the BGE. In Fig. 2, the typical electropherograms of Val in the presence of increasing amounts of potassium ions in the BGE are presented.
2. It is assumed that a 1:1 complex is formed between Val as a ligand (L) and alkali metal or ammonium cation as a central ion (M^+), which can be described by the following equilibrium:

$$M^+ + L \leftrightarrow ML^+ \tag{1}$$

with the appropriate apparent binding constant of this complex, K_{ML}:

$$K_{ML} = \frac{\left[ML^+\right]}{\left[M^+\right]\left[L\right]} \tag{2}$$

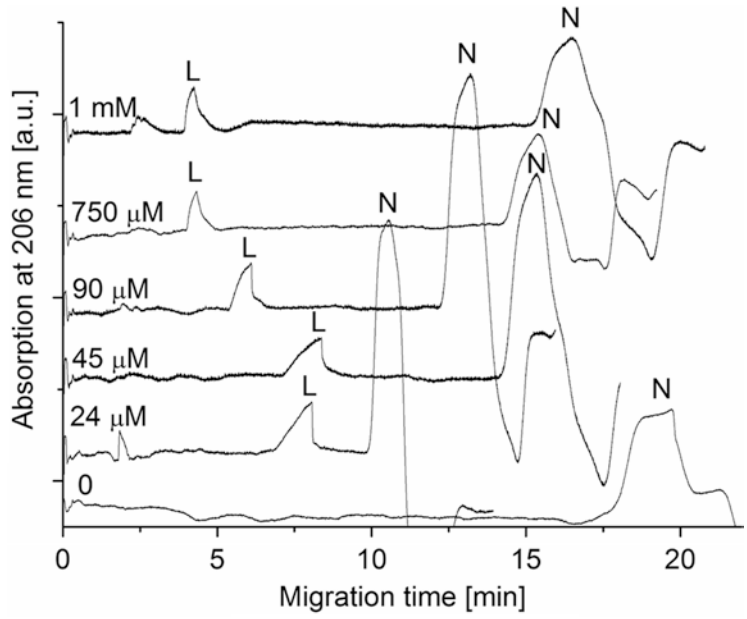

Fig. 2 Typical electropherograms of valinomycin (L) at different concentrations of potassium ions in the BGE (100 mM Tris, 50 mM acetate acid, 0–1 mM KCl). N, neutral EOF marker (DMSO). Adapted with permission from [9]

where $[ML^+]$, $[M^+]$, and $[L]$ are the equilibrium concentrations of the Val–M^+ complex, free alkali metal/ammonium ion, and free Val, respectively. An effective mobility of Val, $m_{L,eff}$, in the presence of M^+ cation can be expressed as follows:

$$m_{L,eff} = m_L \frac{[L]}{[L]+[ML^+]} + m_{ML} \frac{[ML^+]}{[L]+[ML^+]} \tag{3}$$

where m_L and m_{ML} are the electrophoretic mobilities of free Val and Val–M^+ complex, respectively. From Eqs. (2) and (3), the following equation can be obtained for $m_{L,eff}$:

$$m_{L,eff} = \frac{1}{1+K_{ML}[M^+]} m_L + \frac{K_{ML}[M^+]}{1+K_{ML}[M^+]} m_{ML} \tag{4}$$

The free Val mobility, m_L, is zero, because Val is electrically neutral; thus Eq. (4) can be simplified to

$$m_{L,eff} = \frac{K_{ML}[M^+]}{1+K_{ML}[M^+]} m_{ML} \tag{5}$$

The effective mobility of Val, $m_{L,eff}$, at different alkali metal and ammonium ion concentrations in the BGE was calculated from the following equation using the migration times of Val, t_{mig}, and of the EOF marker, t_{eof}, respectively, obtained from the ACE experiments:

$$m_{L,eff} = \frac{L_t L_d}{U}\left(\frac{1}{t_{mig}} - \frac{1}{t_{eof}}\right) \quad (6)$$

where L_t and L_d are the total length of the capillary and the effective capillary length to UV detector, respectively, and U is the applied separation voltage.

3. All ACE experiments should be performed at constant reference temperature or the measured mobilities have to be corrected to this temperature (*see* **Note 1**).
4. Due to the dependence of electrophoretic mobilities on the ionic strength of the BGE, all experiments for binding constant determination should be performed in BGEs with constant ionic strength or the measured mobilities have to be corrected to constant ionic strength (*see* **Note 2**).
5. In order to calculate the binding constants of Val–alkali metal or Val–ammonium ion complexes, the corrected effective mobilities of Val were plotted against the concentrations of M^+ cations in the BGE; *see* Fig. 3. The values of apparent stability constants, K_{ML}, were determined from these dependences by nonlinear regression analysis according to Eq. (5). The decadic logarithmic forms of the apparent stability constants ($\log K_{ML}$) of complexes of Val with ammonium or alkali metal ions in methanol are listed in Table 1.

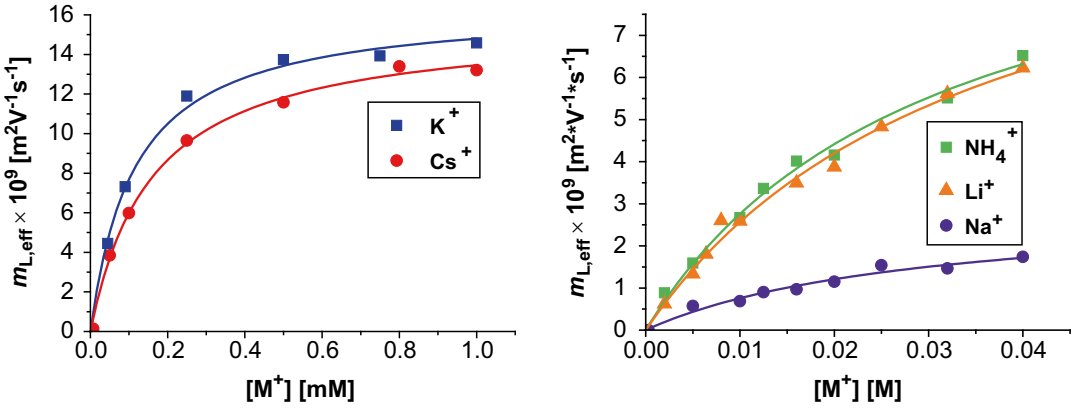

Fig. 3 Dependence of effective mobility of valinomycin, $m_{L,eff}$, on concentration of ammonium or alkali metal ions [M^+] in the BGE (100 mM Tris, 50 mM acetic acid, 0–1 mM KCl or CsCl, or 0–40 mM LiCl, NaCl, or NH_4Cl). Adapted with permission from [9]

Table 1
Logarithm of apparent binding constants, log K_{ML}, or conditional apparent binding constant (log K^{*}_{ML}, *see* Note 3), of Val complexes with ammonium and alkali metal ions in methanol

	log K_{ML} (log K^{*}_{ML})[a]				
Cation	**Our results**	**Literature data [8]**			
NH_4^+	1.52 ± 0.22[b]	1.67[c]			
Li^+	1.50 ± 0.24[a,d]	0.7[c]			
Na+	1.71 ± 0.16[a,e]	0.67[c]	1.08[f]		
K+	3.96 ± 0.29[g]	4.90[c]	>3.9[f]	4.79[h]	4.43[i]
Rb+	4.63 ± 0.27[j]	5.26[c]		5.12[h]	
Cs+	3.81 ± 0.31[g]	4.41[c]		4.32[h]	

[a]Conditional apparent binding constant log K^{*}_{ML}
[b][10]
[c]Spectrophotometry
[d][13]
[e][11]
[f]Potentiometry
[g][9]
[h]Fluorescent spectra
[i]Electric conductivity
[j][12]

4 Notes

1. All ACE experiments were carried out in a homemade device (unequipped with capillary thermostatization) at variable ambient temperature. Therefore, it was necessary to determine the actual temperature of the BGE in the capillary and to correct the measured mobilities to reference temperature 25 °C employing the following procedure. The electric current was measured in a wide range of voltages using the same setup as used for ACE experiments, only with the exception that the capillary was filled with methanolic solution of a strong electrolyte, 0.1 M tetrabutylammonium perchlorate, the electric resistance of which changes with temperature mainly due to the changes of viscosity with temperature. From the measurement of voltage/current ratio the resistance, R, of the electrolyte solution at given input power was calculated. Viscosity, a measure of the internal resistance of a solution, is dependent on the temperature of the solution. According to data [21],

from 20 to 30 °C the viscosity of MeOH decreases 1.34 % *per* °C. In this temperature range, the viscosity of methanolic solution depends linearly on temperature:

$$\eta_T = \eta_{T_0}\left[1 - 0.0134\left(T - T_0\right)\right] \tag{7}$$

where η_T is the viscosity of the electrolyte at given electric input power applied (product of voltage and current), η_{T_0} is the viscosity at the lowest input power applied when the temperature increase in the capillary is taken as negligible, T is the average temperature of the solution inside the capillary, and T_0 is the temperature of surrounding air. Taking into account the 1.34 % decrease in viscosity *per* °C in MeOH, the change of solution resistance at given input power applied was recalculated to the temperature increment, ΔT, occurring at this power:

$$T = \frac{R_0 - R}{0.0134 R_0} \tag{8}$$

where R_0 is the resistance of the solution at the very low input power, when the temperature increase in the capillary can be neglected, and R is the resistance of the solution at given input power applied. Then, a calibration curve of the temperature increase in the solution inside the capillary, ΔT, versus input power applied was obtained. Subsequently the measured effective mobilities were recalculated to the reference temperature of 25 °C, assuming the mean increase of mobility to be 1.34 % *per* °C:

$$m_{\mathrm{eff},25} = m_{\mathrm{eff},T}\left[1 - 0.0134\left(T_0 + \Delta T - 25\right)\right] \tag{9}$$

where $m_{\mathrm{eff},25}$ and $m_{\mathrm{eff},T}$ are the effective mobilities at 25 °C and at actual temperature T inside the capillary, respectively.

2. In case of the strong Val-M^+ complexes (Val-K^+/Rb^+/Cs^+) only small amounts of MCl (up to 1 mM) were added to BGE; thus the ionic strength remained almost constant throughout all the experiments. However, in the case of weak Val-M^+ complexes (Val-Li^+/Na^+/NH_4^+), due to the addition of up to 40 mM concentrations of MCl to the BGEs, the mobilities obtained in these BGEs and corrected to reference temperature 25 °C were recalculated to the values corresponding to the constant ionic strength. For mobility corrections, the extended Debye, Hückel, and Onsager (DHO) theory introduced by Falkenhagen et al. [22] and Pitts [23] was employed. It describes the dependence of the ionic mobility of a uni-univalent electrolyte on the ionic strength:

$$m = m_0 - \left[\frac{8.204 \times 10^5}{(\varepsilon_r T)^{3/2}} m_0 + \frac{4.275}{\eta (\varepsilon_r T)^{1/2}} \right] \times \frac{\sqrt{I}}{1 + 50.29 a (\varepsilon_r T)^{-1/2} \sqrt{I}} \quad (10)$$

where m_0 is the mobility at zero ionic strength (10^{-9} m^2/V/s), $\varepsilon_r = \varepsilon / \varepsilon_0$ is the relative permittivity of the solvent (the ratio of the absolute permittivity ε to that of vacuum ε_0), η is the solvent viscosity (Pa s), T is the absolute temperature (K), and a (in Å, 1 Å = 0.1 nm) is the ion size parameter. After the insertion of the physical parameters for the pure methanol at 25 °C ($\varepsilon_r = 32.63$, $\eta = 0.000544$ Pa s) to Eq. (10) the following equation is obtained:

$$m = m_0 - [0.855 m_0 + 79.67] \times \frac{\sqrt{I}}{1 + 0.510 a \sqrt{I}} \quad (11)$$

The mobility values obtained in BGEs of various ionic strengths were recalculated to constant ionic strength employing Eq. (11); the value of 15.1 Å for ion size parameter a was taken from literature [24] where it was used for tetraphenylphosphonium ion with similar dimension and low electrical charge density. Significant influence of both temperature and ionic strength corrections on the effective mobility of Val is demonstrated in Fig. 4.

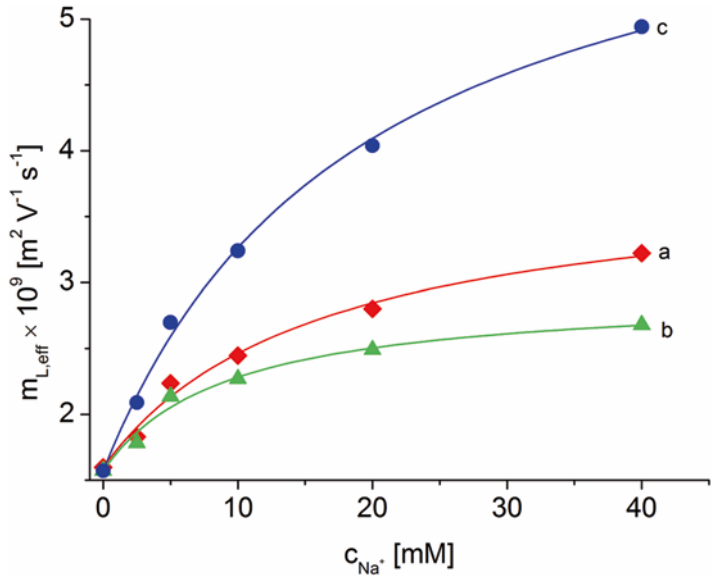

Fig. 4 Dependence of the effective mobility of valinomycin, $m_{L,eff}$, on sodium ion concentration, c_{Na^+}, in the BGE (10 mM Tris, 20 mM chloroacetic acid, 0–40 mM NaCl). Effective mobilities (**a**) at actual temperature and actual ionic strength of the BGE; (**b**) corrected to reference temperature 25 °C; (**c**) corrected to reference temperature 25 °C and constant ionic strength 10 mM. Adapted with permission from [11]

3. When studying Val complexes with Na^+ and Li^+ ions in 10 mM Tris and 20 mM chloroacetate BGE we observed that Val interacts not only with these ions but also with $Tris^+$ cation of the BGE. Na^+ or Li^+ and $Tris^+$ cations react with Val competitively as Val can bind only one of them at a time into its size-limited cavity:

$$Na^+ + L \leftrightarrow NaL^+ \tag{12}$$

$$Tris^+ + L \leftrightarrow TrisL^+ \tag{13}$$

The corresponding equilibrium apparent binding constants are

$$K_{NaL} = \frac{\left[NaL^+\right]}{\left[Na^+\right][L]} \tag{14}$$

$$K_{TrisL} = \frac{\left[TrisL^+\right]}{\left[Tris^+\right][L]} \tag{15}$$

where $[NaL^+]$, $[Na^+]$, $[TrisL^+]$, $[Tris^+]$, and $[L]$ are the equilibrium concentrations of the Val–Na^+ complex, free Na^+ ion, Val-Tris complex, free $Tris^+$ ion, and free Val, respectively. The Val effective mobility in the presence of Na^+ and $Tris^+$ ions, $m_{L,eff}$, can be described by the following equation [25]:

$$m_{L,eff} = \frac{m_L + K_{NaL}\left[Na^+\right]m_{NaL} + K_{TrisL}\left[Tris^+\right]m_{TrisL}}{1 + K_{NaL}\left[Na^+\right] + K_{TrisL}\left[Tris^+\right]} \tag{16}$$

where m_L, m_{NaL}, and m_{TrisL} are the electrophoretic mobilities of free Val, Val–Na^+, and Val–$Tris^+$ complexes, respectively. When $[Tris^+]$ is constant, Eq. (16) can be simplified to

$$m_{L,eff} = \frac{m_L^* + K_{NaL}^*\left[M^+\right]m_{NaL}}{1 + K_{NaL}^*\left[M^+\right]} \tag{17}$$

where m_L^* is the effective mobility of Val in the BGE in the absence of Na^+ ions, and K_{NaL}^* is the conditioned apparent binding constant of the NaL^+ complex in the presence of $Tris^+$ cation. Nonzero value of m_L^* results from the interaction between Val and $Tris^+$ and its value was determined from the Val analysis performed in the BGE, which did not contain any Na^+ or Li^+ ions.

The conditioned apparent binding constants of Val-Na^+ and Val-Li^+ complexes (K_{ML}^*) were determined from the dependences

of electrophoretic mobilities of Val on the Na^+ or Li^+ ion concentrations in the BGE by nonlinear regression analysis according to Eq. (17) and are given in Table 1.

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References

1. Gouaux E, MacKinnon R (2005) Principles of selective ion transport in channels and pumps. Science 310:1461–1465
2. Shirai O, Yoshida Y, Kihara S (2006) Voltammetric study on ion transport across a bilayer lipid membrane in the presence of a hydrophobic ion or an ionophore. Anal Bioanal Chem 386:494–505
3. Rose L, Jenkins ATA (2007) The effect of the ionophore valinomycin on biomimetic solid supported lipid DPPTE/EPC membranes. Bioelectrochemistry 70:387–393
4. Varma S, Sabo D, Rempe SB (2008) K+/Na + selectivity in K channels and valinomycin: Over-coordination versus cavity-size constraints. J Mol Biol 376:13–22
5. Pressman BC (1976) Biological Applications of Ionophores. Annual Review of Biochemistry 45:501–530
6. Ovchinnikov YA (1979) Physicochemical Basis of Ion-Transport Through Biological-Membranes - Ionophores and Ion Channels. Eur J Biochem 94:321–336
7. Wang F, Zhao CX, Polavarapu PL (2004) A study of the conformations of valinomycin in solution phase. Biopolymers 75:85–93
8. Izatt RM, Bradshaw JS, Nielsen SA et al (1985) Thermodynamic and Kinetic Data for Cation Macrocycle Interaction. Chem Rev 85: 271–339
9. Ehala S, Kašička V, Makrlík E (2008) Determination of stability constants of valinomycin complexes with ammonium and alkali metal ions by capillary affinity electrophoresis. Electrophoresis 29:652–657
10. Dybal J, Ehala S, Kašička V et al (2008) Theoretical and Experimental Study of the Complexation of Valinomycin with Ammonium Cation. Biopolymers 89:1055–1060
11. Ehala S, Dybal J, Makrlík E et al (2009) Capillary affinity electrophoresis and ab initio calculation studies of valinomycin complexation with Na + ion. J Sep Sci 32:597–604
12. Ehala S, Dybal J, Makrlík E et al (2009) Capillary electrophoretic and computational study of the complexation of valinomycin with rubidium cation. Electrophoresis 30:883–889
13. Ehala S, Dybal J, Makrlík E et al (2009) Application of capillary affinity electrophoresis and density functional theory to the investigation of valinomycin-lithium complex. J Chromatogr A 1216:3660–3665
14. Chen Z, Weber SG (2008) Determination of binding constants by affinity capillary electrophoresis, electrospray ionization mass spectrometry and phase-distribution methods. Trends Anal Chem 27:738–748
15. Ostergaard J, Jensen H, Hoim R (2009) Use of correction factors in mobility shift affinity capillary electrophoresis for weak analyte - ligand interactions. J Sep Sci 32:1712–1721
16. Jiang CX, Armstrong DW (2010) Use of CE for the determination of binding constants. Electrophoresis 31:17–27
17. Dvořák M, Svobodová J, Beneš M et al (2013) Applicability and limitations of affinity capillary electrophoresis and vacancy affinity capillary electrophoresis methods for determination of complexation constants. Electrophoresis 34:761–767
18. Porras SP, Riekkola ML, Kenndler E (2001) Capillary zone electrophoresis of basic analytes in methanol as non-aqueous solvent - Mobility and ionisation constant. J Chromatogr A 905:259–268
19. Beckers JL, Ackermans MT, Boček P (2003) Capillary zone electrophoresis in methanol: Migration behavior and background electrolytes. Electrophoresis 24:1544–1552
20. Kašička V, Prusík Z, Sázelová P et al (1999) Capillary zone electrophoresis with electroosmotic flow controlled by external radial electric field. Electrophoresis 20:2484–2492

21. Papanastasiou GE, Ziogas II (1992) Physical Behavior of Some Reaction Media. 3. Density, Viscosity, Dielectric-Constant, and Refractive-Index Changes of Methanol + Dioxane Mixtures at Several Temperatures. Journal of Chemical and Engineering Data 37:167–172
22. Falkenhagen H, Leist M, Kelbg G (1952) *Zur Theorie der Leitfahigkeit Starker Nicht Assoziierender Elektrolyte Bei Hoheren Konzentrationen. Annalen der Physik 11: 51–59
23. Pitts E (1953) An Extension of the Theory of the Conductivity and Viscosity of Electrolyte Solutions. Proceedings of the Royal Society of London Series A-Mathematical and Physical Sciences 217:43–70
24. Muzikář J, van de Goor T, Gaš B et al (2002) Electrophoretic mobilities of large organic ions in nonaqueous solvents: Determination by capillary electrophoresis in propylene carbonate, N, N-dimethylformamide, N, N,-dimethylacetamide, acetonitrile and methanol. Electrophoresis 23:375–382
25. Peng XJ, Bowser MT, Britz-McKibbin P et al (1997) Quantitative description of analyte migration behavior based on dynamic complexation in capillary electrophoresis with one or more additives. Electrophoresis 18:706–716

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