APPLICATIONS OF CAPILLARY ELECTROPHORESIS -MASS SPECTROMETRY INTERFACED BY A FLOW-THROUGH MICROVIAL ELECTROSPRAY IONIZATION SPRAYER

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ABSTRACT

Capillary electrophoresis – electrospray ionization – mass spectrometry (CE-ESI-MS) combines the superior separation capability of CE and detection and characterization ability of MS. Different CE separation modes can be coupled to ESI-MS, employing an interface with a flow-through microvial. In the first part of the thesis, recent development of CE and CE-MS applications in the analysis of complex samples are reviewed.

Capillary isoelectric focusing (cIEF) is an important tool for the separation and characterization of amphoteric molecules based on isoelectric points. Minute structural changes on a large protein can result in changes in isoelectric point, and the changes can be detected by slab gel isoelectric focusing or capillary isoelectric focusing. A systematic study on the interactions among carrier ampholytes, sample media and capillary inner coatings was carried out to provide guidelines for choosing feasible combinations that can achieve isoelectric focusing and successful chemical mobilizations. within the 0.1%-1% (w/v) carrier ampholytes concentration range, small forward EOFs will ensure a higher chance of good focusing and successful electrophoretic mobilization, while a negative EOF will hinder these processes. Feasible combinations of experimental conditions are summarized. Using the optimized conditions, we reported the direct

observation of the shape of focused ampholyte bands in the cIEF process by online cIEF-ESI-MS. The ampholyte bands directly detected by MS have the potential to enable a more accurate pI determination for unknown amphoteric molecules. Immunoglobulin G from rabbit serum is used to demonstrate this possibility.

In Chapter 6, a CE-MS method was developed to monitor the concentration variations of major nutrients and/or metabolites in human embryonic stem cell CA1S culture medium over a culturing cycle. Concentration changes for nutrients and/or metabolites in the culturing media provided information on the cell growth behavior without destructing living cells.

In the last part of the thesis, an atmospheric ion lens was applied to the flow-through microvial CE-ESI-MS interface to improve the electrospray ionization and sampling efficiency. A mixture of amino acids was tested to show the increased signal-to-noise ratios. The atmospheric ion lens also gives more flexibility when choosing the EOF and chemical modifier flow rates.

PREFACE

The majority of the research included in this dissertation was conducted by the author, Shuai Sherry Zhao. The contributions of other researchers and collaborations are detailed below.

Contributions from other researchers:

Chapter 3: The glycan analysis literature was summarized by Cai Tie, and the capillary isoelectric focusing literature was summarized by Xuefei Zhong.

Chapter 4: The EOF calculations were performed by Alexis Lee.

Chapter 6: The cell culture was performed collaboratively with Chris Sherwood, and James Piret provided the lab space and supplies for the culture of CA1S cell line. The metabolite standards were provided from David Wishart's research group of University of Alberta.

Chapter 7: The computer simulation was performed collaboratively with Xuefei Zhong.

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Material from this article is included in Chapter 2.

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Material from this article is included in Chapter 7.

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LIST OF ABBREVIATIONS

APCI	atmospheric pressure chemical ionization
2DE	two dimensional gel electrophoresis
APPI	atmospheric pressure photoionization
BGE	background electrolyte
CAs	carrier ampholytes
CE	capillary electrophoresis
CE-SDS	sodium dodecyl sulfate capillary gel electrophoresis
cIEF	capillary isoelectric focusing
CZE	capillary zone electrophoresis
DC	direct current
EOF	electroosmotic flow
ESI	electrospray ionization
FASS	field-amplified sample stacking
GC	gas chromatography
G-CSF	granulocyte-colony stimulating factor
hESCs	human embryonic stem cell
HPLC	high performance liquid chomatograpy
icIEF	imaging cIEF
ID	inner diameter
IEF	isoelectric focusing
IT	ion trap
MALDI	matrix assisted laser desorption ionization
MS	mass spectrometry
MS^n	tandem mass spectrometry
MW	molecular weight
Myo	myoglobin
OD	outer diameter
PEG	polyethyleneglycol
PEI	polyethyleneimine
PTMs	post-translational modifications
PVA	polyvinyl alcohol
QQQ	triple quadrupole mass analyzer
RF	radio frequency
rhEPO	recombinant human erythropoietin
RNase	ribonuclease A

SPE	solid phase extraction
t-ITP	transient isotachophoresis
TOF	time-of-flight
UV	ultra-violet
β-lac	β-lactoglobulin

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Chapter 1. Introduction to capillary electrophoresis and mass spectrometry coupling

1.1.Capillary electrophoresis

The groundwork for the analysis of diverse analytes, ranging from small molecules to proteins and viruses, by capillary electrophoresis was laid by Hjerten and coworkers' pioneering work approximately half a century ago. [1] In 1981, the spectacular separations of peptides were carried out by Jorgenson and Lucas using zone electrophoresis, defining electrophoresis in micron-scale capillaries. [2] Featuring high resolution, versatility and high speed, CE made its way into the research community and into industry.

High performance liquid chromatography (HPLC), an alternative to CE, has also made automation, high speed, high resolution, and reliable quantification possible during the same period. [3] However, with HPLC, the analysis of large proteins is problematic due to column clogging. Moreover, the significant organic solvent consumption associated with the use of HPLC is also a disadvantage, while CE analysis is mostly carried out in small volumes of aqueous solutions. [4] CE is a great complementary technique to HPLC, because it not only solves the column-clogging and solvent-consumption problems, but also provides orthogonal information of analytes based on their charge properties.

1.1.1. Instrumentation

The instrumentation of CE is surprisingly simple. Figure 1.1 shows the

schematic set-up for a CE apparatus. This design depicts a home-made instrument and the core structure for commercially available systems with on-column optical detection. It consists of a separation capillary with two ends inserted into buffer vials. The capillary is usually made of fused silica coated with polyimide on the outside, and has an inner diameter smaller than 200 μm. Close to the end (or outlet) of the capillary, a small portion of the polyimide is removed to create a transparent light path for optical detection which is called the detection window. A very high voltage is applied across the capillary by a power supply.

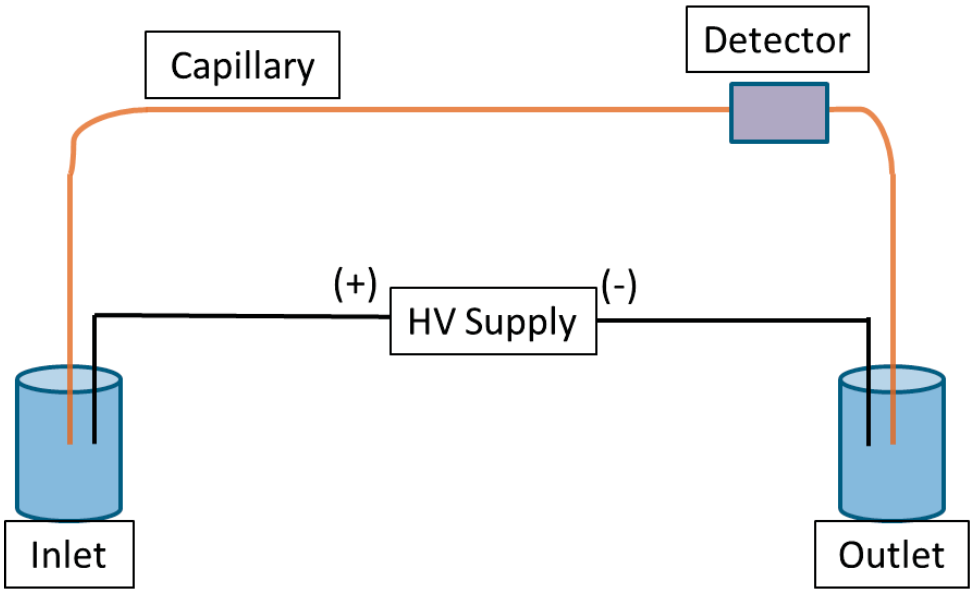

Figure 1.1 CE instrument set-up

There are several general steps for a CE analysis. The capillary column is pre-equilibrated by flushing it with background electrolyte (BGE) prior to sample injection. A small plug of sample, which usually occupies less than 3% of the total capillary column unless special pre-concentration techniques are performed, is

injected into the capillary column, either hydrodynamically or electrokinetically. A high voltage is then applied to drive the species towards the CE outlet. The analytes, separated based on their different mobilities in the BGE, pass by the detection window, causing optical absorbance changes at different time. The changes can then be converted into digital signals and recorded by a computer. [5]

1.1.2. Capillary zone electrophoresis

Capillary zone electrophoresis (CZE) is the simplest and most often used separation mode. It is carried out in free solutions and separates analytes based on their electrophoretic mobilities which are determined by their charge to size ratios. [6]

The normal polarity of CZE is considered to be from the anode (inlet) to the cathode (outlet and detector). Upon applying a high voltage across the separation capillary, the charged species in the solution experience the electrostatic force $\vec{F}_e$ (1-1) which causes the ions to accelerate. The moving species also experience a drag force $\vec{F}_D$ (1-2) from the solution. Since the $\vec{F}_D$ (1-2) is proportional to the velocity and it soon balances $\vec{F}_e$ (1-3), the ion travels at constant electrophoretic velocity $\vec{v}_{ep}$ (1-4) determined by the ion's net charge q, the electric field in the capillary $\vec{E}$, the hydrated ion radius R, and the viscosity of the solution η.

$$\vec{F}_e = q\vec{E} \tag{1-1}$$

$$\vec{F}_D = 6\pi\eta r\vec{v} \tag{1-2}$$

$$q\vec{E} = 6\pi\eta r\vec{v} \tag{1-3}$$

$$\vec{v}_{ep} = \frac{q\vec{E}}{6\pi\eta R} \tag{1-4}$$

The electrophoretic mobility, independent from electric field, is defined below (1-5) to describe an ion's electro-migrational behavior. It is only determined by the ion's charge-to-size ratio in free solution. In CZE, analytes are separated based on their electrophoretic mobilities. [7]

$$\vec{\mu}_{ep} \equiv \frac{\vec{v}_{ep}}{\vec{E}} = \frac{q}{6\pi\eta R} \tag{1-5}$$

Clearly, from Eq (1-5), the neutral species have a zero electrophoretic mobility and negative ions move towards the inlet for the set-up shown in Figure 1.1. Should the transportation through the capillary be driven solely by the individual electrophoretic mobilities, not all of the species would pass through the detection window. However, under appropriate conditions, all analytes can be detected, indicating that there is another bulk flow that drives the analytes in the separation capillary from the anode to the cathode, which is called electroosmotic flow (EOF). [8]

Coulombic force, which is induced by the electric field on the net mobile charges in the solution, causes EOF in the capillary column. Taking the commonly used bare fused silica capillary as an example, the ionized silanol groups on the inner wall attract cationic species from the buffer and chemical equilibrium leads to a tight knit fixed electrical charge layer (Stern layer) and a layer of mobile ions (outer Helmholtz plane). The positive charge density decreases exponentially here with the increase of distance from the capillary wall. [9] The electrical double layer is shown diagrammatically in Figure 1.2. When an electric field is applied to the fluid, the net charge in the outer Helmholtz plane, composed of cations in this case, is induced by

Coulombic force to migrate towards the cathode, carrying hydrating water molecules with them. The cohesive hydrogen bonding between hydrating water molecules and bulk solution water molecules causes the entire solution to be dragged towards the cathode. Thus forms the EOF, with a flat flow profile resulted from applying a voltage along the capillary.

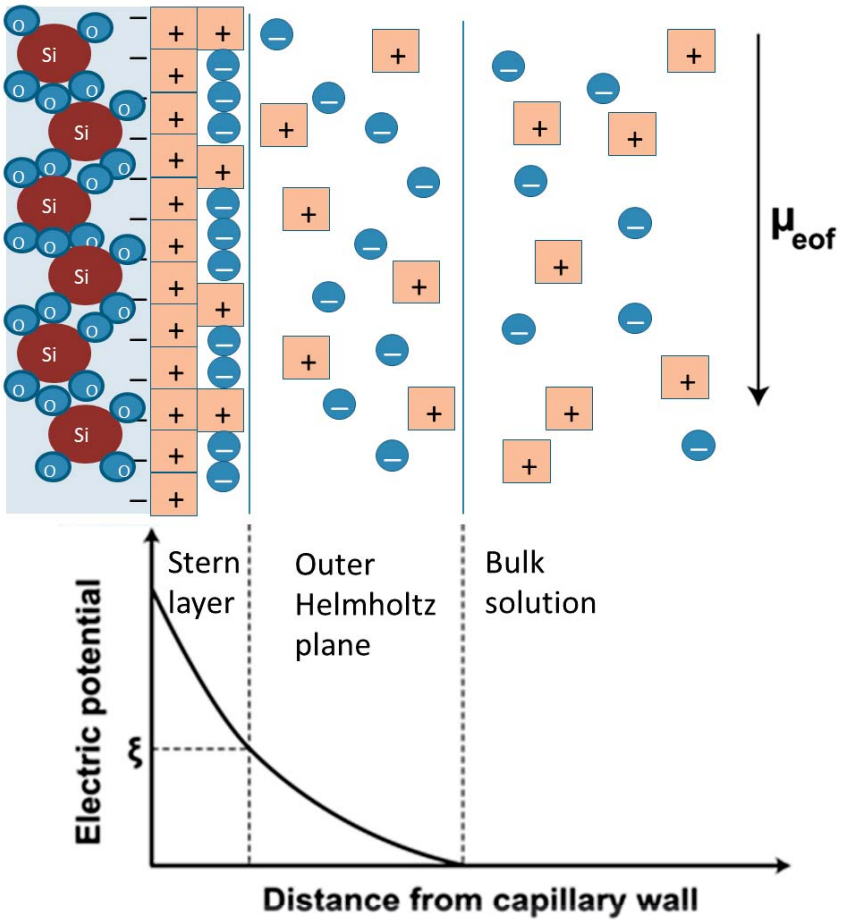

Figure 1.2. Schematic diagram of electrical double layer

Unlike a pressure-driven parabolic flow, EOF's velocity profile is almost planar, with only slight variations caused by frictional forces near the solid-liquid interface. Thus, EOF is non-discriminative and offers substantially less dispersive effects for the separation process.

Since the dissociation of the silanol groups are determined by temperature and buffer pH, EOF is also related to the two factors.The EOF magnitude is determined by Eq (1-6), where ε is the dielectric constant of the fluid, ζ is the zeta potential, η is the visocity of the fluid. [10]

$$\vec{\mu}_{eo} = \frac{\vec{v}_{eo}}{\vec{E}} = \frac{\varepsilon\zeta}{4\pi\eta} \qquad (1\text{-}6)$$

The apparent electrophoretic mobility is the sum of electroosmotic mobility and electrophoretic mobility (1-7). Analytes with different apparent electrophoretic mobilities are separated under CZE modes, as depicted in Figure 1.3. [11]

$$\vec{\mu}_{app} = \vec{\mu}_{ep} + \vec{\mu} \qquad (1\text{-}7)$$

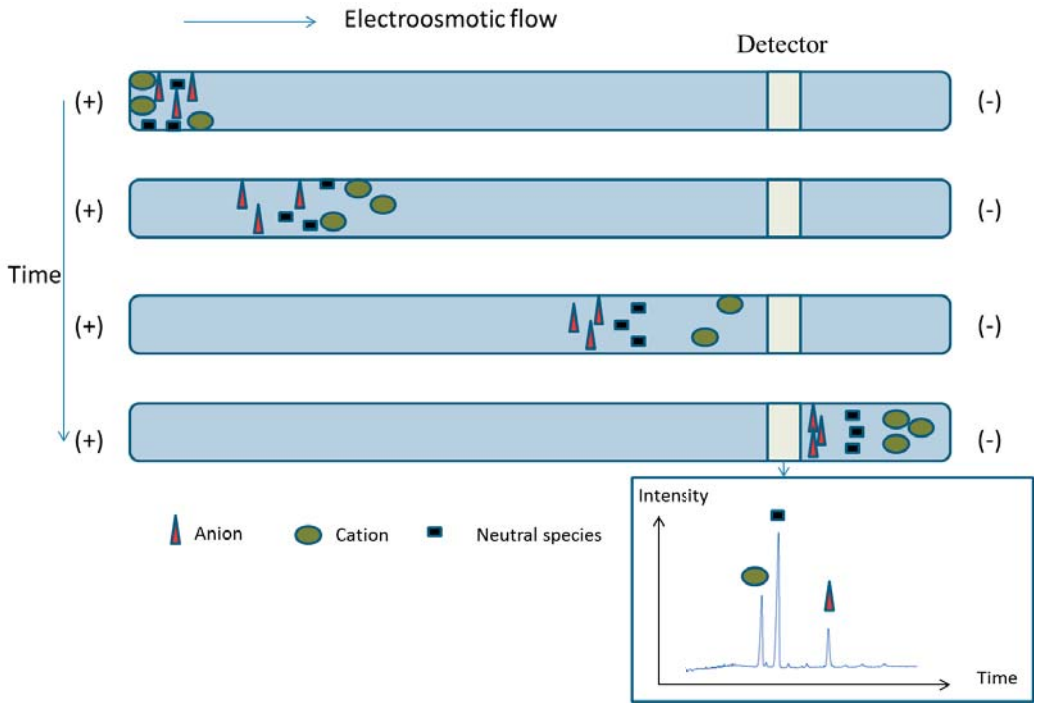

Figure 1.3. Schematic CE separation

1.1.3. Other separation modes

1.1.3.1.Capillary isoelectric focusing

Isoelectric focusing (IEF) is a widely used electrophoretic technique for amphoteric molecule separation and characterization based on isoelectric point (pI). Minute changes on a large protein, such as phosphorylation, glycosylation, and other types of post-translational modifications, can change its pI, and thus the isoforms can be separated by IEF. Effective IEF was not accomplished until the late 1960s when Vesterberg synthesized ampholine to create a stable pH gradient. [12] This slab-gel formatted analytical technique has been used ever since as one of the routine tools for biochemical and biomedical analysis for complex protein mixture as well as for characterizations of purified protein. [13] Slab gel IEF, as a major protein characterization tool, is labor intensive, time consuming and incapable of

quantification. In 1985, Hjerten and Zhu performed IEF in a CE set-up and laid the ground work for capillary isoelectric focusing (cIEF). [14] This alternative method maintains the high separation power based on pI while reducing the consumption of sample, material, labor, and time. Another benefit of cIEF is the possibility of protein quantification using the CE detection system.

cIEF experimental set-up usually includes a basic catholyte at the cathode and an acidic anolyte at the anode. A mixture of the protein to be analyzed and carrier ampholytes fills up the majority of the separation capillary. A protein becomes positively charged when its pI value is higher than that of the local pH, and negatively charged in the opposite situation. When an electric field is applied to the capillary, the protein will migrate towards the oppositely charged electrode. As the protein migrates along the pH gradient, the charge on the protein will decrease to zero when it reaches the region where the local pH is the same as its own pI. At the pI, the electrophoretic mobility of the particle should be zero, so that proteins, which fill up the whole capillary at the beginning, are focused into sharp bands according to their pIs in a pH gradient along the capillary. [15]

It should be noted that there are two additional requirements for a successful focusing process: zero or substantially reduced sample-capillary wall interaction, and no or minimum bulk flow, including EOF flow and hydrodynamic flow. The negatively charged silanol inner surface of a bare fused silica capillary is never an optimal non-interaction capillary wall for protein analysis, especially for cIEF; that is why most cIEF experiments are carried out with neutrally coated capillaries as this

reduces the sample-capillary wall interaction. [16,17] The capillary inner wall modification also allows EOF magnitude to subside. Another way to reduce EOF bulk flow is to add viscous anti-convective additives and medium to the sample mixture, which has been applied routinely in cIEF analysis. [18] To maintain no hydrodynamic flow, the two ends of the separation capillary should be kept at the same level. [19]

After the sample mixture has finished focusing, denoted by a minimum current, the separated amphoteric molecules can be detected either through the whole-column or at a single single-point. Whole-column detection cIEF or imaging cIEF (icIEF) allows the *in situ* monitoring of focusing process and avoids the mobilization of the (near-) stationary focused sample train. The detection methods include UV-Vis [20], fluorescence [21], chemiluminescence [22]. icIEF has been used extensively in the charge variants monitoring for the recombinant therapeutic protein production. [23]

Single-point detection cIEF, which is most commonly used with commercial CE instruments, requires focused protein bands to pass through the detection window. Therefore, a mobilization step for the protein train is needed. This can be achieved hydrodynamically [16], electroosmotically [18], or electrophoretically [14]. The simplest method is to use a pressure-driven or gravity-driven hydrodynamic flow, either applying a small pressure at one end of capillary or raising the inlet end of the capillary to create a height difference. [24-26] The advantages are its simplicity and the preservation of the pH gradient linearity formed during focusing. However, the laminar flow in this situation could deteriorate resolution obtained from the focusing step. In electroosmotic mobilization or EOF mobilization, the EOF of capillary is not

totally eliminated but well controlled. Focusing and mobilization driven by EOF happen concurrently. This is usually observed in cIEF with bare fused silica columns. [27] Because of the changes in pH and field strength across the capillary, it is difficult to control the EOF during the whole process. Electrophoretic mobilization, also referred to as chemical/anion/cation/salt mobilization, is to substitute the terminal electrolyte with another one after focusing to induce the mobilization under electric field. [28,29] No parabolic flow is introduced in this approach, and resolution achieved during focusing is maintained. However, the linearity between pIs and migration times may not be as well maintained as that with hydrodynamic mobilization. Each of the three mobilization methods, or combinations of the three, has been used by researchers. [17]

1.1.3.2.Other major capillary electrophoretic separation modes

In addition to CZE and cIEF, several other separation modes can be carried out using the same CE hardware. Depending on the analytes' physical or chemical properties, simply changing the buffer and/or separation capillary can achieve different separation methods. The major ones include, but are not limited to, micellar electrokinetic chromatography (MEKC or MECC) [30-32], capillary electrochromatography (CEC) [33,34], capillary gel electrophoresis (CGE) [35,36] and capillary isotachophoresis (CITP) [37,38]. The following table summarizes the general principles, capillary types, buffer compositions, and common applications of these methods.

Table 1.1. Summarization of Major CE Separation Modes

	Principle	Treatment on Capillary	Buffer Composition	Application
MEKC	Micellar pseudostationary phase interacts with solutes according to partition mechanisms	No special treatments required	Surfactant above its CMC* added to buffer; organic solvents added to enhance the mobility	Small molecules; peptides
CEC	Chromatographic separation mechanisms	Containing chromatographic stationary phases	Appropriate mobile phase for analytes	Small molecules; peptides; proteins; carbohydrates
CGE	Sieving mechanisms according to size	Gel covalently bond to capillary	Polyacrylamide or hydroxyalkyl celluloses gel filled	Proteins, DNA sequencing and DNA fragment mapping
CITP	Ionic compounds migrating at the same velocity between on highest-mobility (leading) electrolyte and lowest-mobility (terminating) electrolyte	No special treatments required	Discontinuous buffer system with one high-mobility buffer and the other low-mobility one	Ionic compounds; sample preconcentration

*CMC, critical micelle concentration

1.2.Mass Spectrometry

Mass spectrometry (MS) analyzes gaseous ions based on mass-to-charge ratios (m/z). It can, with high sensitivity, high speed, and versatility, measure the mass of positively or negatively charged ions, provide the elemental composition of a compound, and elucidate the chemical structure by performing fragmentation. The general steps of a MS method involve ionizing the chemicals in the ion source to

generate charged molecules or charged fragments, transferring the charged particles via ion optics, analyzing the m/z by mass analyzer and performing fragmentation (MS^n), detecting the ions by detector, and converting detected signals into readable forms by computer. [5]

The diagrammatic set-up of a mass spectrometer is provided in Figure 1.4.

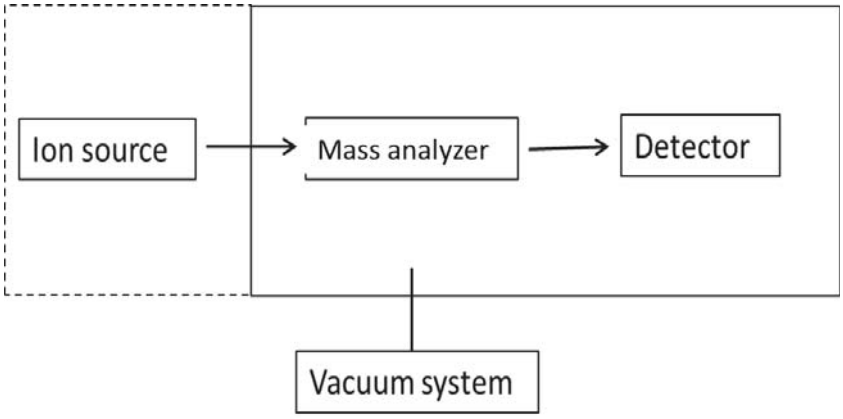

Figure 1.4. General set-up for a mass spectrometer

1.2.1. Ionizations

The primary step for MS analysis is to generate charged particles from the sample medium, which can be in gas, liquid or solid phase. Associated with the development of MS technology, are a lot of ionization methods that have been invented and applied. The common ionization techniques which can be coupled to CE analysis include inductively coupled plasma ionization [39], atmospheric pressure chemical ionization [40], atmospheric pressure photonionization [41,42], electrospray ionization (ESI) , and matrix-assisted laser desorption ionization [43,44]. In this chapter, only ESI is discussed in detail because of its relevance to the work presented in this thesis.

ESI transfers charged species from liquid phase to gas phase under atmospheric

pressure. It is a soft ionization technique, which means charged species do not undergo extensive fragmentation, in contrast to earlier-developed hard ionization techniques such as electron impact ionization. [45] Before introduced to MS, electrospray had already been widely used in ink-jet printing, crop spraying, and paint spraying for generating fine aerosols. [46] It was not until 1968 that Dole first attempted to use electrospray to generate gas-phase macromolecule ions from solutions. [47] Two decades later, Fenn and Yamashita successfully coupled ESI to MS. [48] Ever since then, ESI has seen its great prosperity in applications ranging from small molecules to large polymers. In the study of biomolecules, ESI plays an extremely important role, and has been applied in proteomics [49], metabolomics [50], glycomics [51], lipidomics [52], and non-covalent biomolecule interactions and complexations [53]. Meanwhile, the liquid-gas-transfer ability of ESI makes it a great candidate for HPLC-MS and CE-MS online couplings.

Three general steps are involved in ESI to produce gas-phase ions out of liquid: charged droplets formation by high electric field at electrospray tip, charged droplets shrinkage by solvent evaporation and Coloumbic fission, and gas-phase ion production. [54]

To pull out charged droplets requires the electric field at the electrospray tip to overcome the surface tension of the bulk solution. A typical positive mode ESI set-up is shown in Figure 1.5. The electrospray tip is usually a metal capillary with a small outer diameter (i.e. several hundreds of microns) and the potential difference between it and the mass spectrometer sampling inlet is usually between 2-5 kV. The distance

between the two is usually about 1 cm, so the electric field at the electrospray tip is ~10^6 V/m. The high electric field exerted on the positive ions causes the bulk solution coming out of the electrospray tip (typically at a flow rate of 1-20 μL/min) to stretch downfield. A liquid cone, which is commonly called the Taylor cone, forms at the tip because of the high electric field while the surface tension of the bulk solution contracts the cone inwards. When the electric field is high enough to overcome the surface tension, a fine jet will form from the Taylor cone tip, which breaks into smaller charged droplets. For each electrospray tip, as well as solution composition, there is a threshold for the electric field, called the 'on-set electric field'. [55] The size of the charged droplet is mainly determined by the diameter of electrospray tip orifice: the smaller the tip orifice, the finer the droplet size. At atmospheric pressure, on the path towards the counter electrode, i.e. the mass spectrometer sampling inlet, the charged droplets shrink in size. Two factors contribute to the shrinkage; one factor is the solvent evaporation and the other is the mutual Coulombic repulsion of ions contained in the droplets overcoming surface tension of the droplets and causing the droplet fragmentation. The latter is called Coulombic fission, and finer offspring droplets are generated. [56,57]

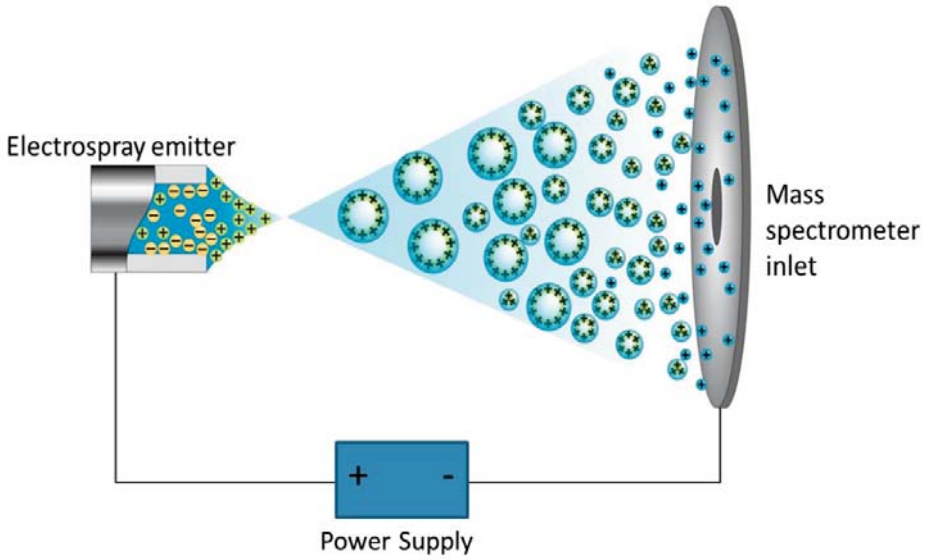

Figure 1.5. A typical positive ESI illustration
A positive high voltage is applied on the electrospray emitter, through which the solution containing analyte is pumped. A Taylor cone is formed at the emitter tip because of the electric field. When the electric field overcomes the surface tension, charged droplets form, which later undergo solvent evaporation and Coulombic fission to form gaseous ions. The ions are sampled into the mass spectrometer inlet and analyzed according to their mass-to-charge ratios.

From the small and highly charged droplets, gas-phase ions form. Two possible mechanisms account for this formation. The charged residue mechanism (CRM), proposed by Dole [47], suggests that when the solution is dilute enough, the droplets formed from fissions will be so small that each one will only contain one ion. After the solvent evaporates, the solute will be free and bear charges. The ion evaporation mechanism (IEM) [58,59] predicts that direct ion emission from the droplets will happen if the droplet size is small enough.

1.2.2. Mass Analyzers

Mass analyzer differentiates between ions based on their m/z ratios; thus it is one of the core components of a mass spectrometer. In this section, three types of mass analyzers are discussed and are used in later applications either on their own or in

combination with one another. The mass analyzers included in this chapter are time-of-flight (TOF), quadrupole and 3D ion trap (IT).

1.2.2.1.Time-of-flight mass analyzer [60]

TOF mass analyzers employ a simple concept for m/z discrimination: if there is no field applied in a fixed-length tube in vacuum, faster ions will travel a shorter time to reach the destination. To obtain an initial energy, ions travel through an electric field, where U is the acceleration potential, before entering the field-free drift tube. For a charged particle with almost zero initial velocity, the final velocity, i.e. the velocity at which the ion enters the field-free drift tube, can be calculated by Eq (1-8). The drift time is equal to the length divided by the velocity and can be related to its m/z, according to Eq (1-9), where *t* is the drift time, and *L* is the length of the drift tube.

$$\mathrm{Uze} = \frac{1}{2}\mathrm{m}v^2 \qquad (1\text{-}8)$$

$$\frac{m}{z} = \frac{2eUt^2}{L^2} \qquad (1\text{-}9)$$

Ions with same m/z may end up with different initial kinetic energies from the ion source entering the field-free tube. To correct the energy dispersion, a reflectron is usually added. A reflectron is composed of a series of grid electrodes at the other end of the field-free tube, opposing the entrance. The detector is placed at the side of the entrance. When ions enter the reflectron region, the electric field will decelerate ions and eventually reverse their travelling directions. Ions with more kinetic energy will penetrate further into the reflectron, compensating for the shorter time they spend in

the drift tube. By applying proper potentials on the grid electrodes, ions with the same m/z will arrive at the detector at almost the same time.

In principle, there is no upper limit of the mass range for a TOF mass analyzer, since the larger ions would just spend more time in the drift tube. This makes TOF ideal for large biomolecule analysis. Also, the accelerated ions are injected into the drift tube together in a pulse, not continuously. This feature provides easy coupling between TOF and MALDI where the ions are produced in a pulse at the ion source. MALDI is such a prevalent ionization technique for large biomolecules that MALDI-TOF coupling is almost a standardized analytical tool for proteins in both the research community and industry. Another advantage of TOF is the speed at which a broad mass range spectrum may be generated; a complete spectrum may be completed in a few hundred micro seconds.

1.2.2.2.Quadrupole mass analyzer [61]

As implied by the name, a quadrupole mass analyzer consists of four rod electrodes which are set parallel to each other, shown diagrammatically in Figure 1.6. Ideally the rods are hyperbolic, but circular rods with a satisfactory diameter-to-distance-between-rods ratio also provide good m/z differentiation, while being easier to manufacture. Therefore, commercial mass spectrometers have been employing both types of rods. The rods are grouped into two pairs, with each pair containing two opposing rods connected electrically. Between the two pairs, a radio frequency (RF) voltage is applied and a direct current (DC) voltage is superimposed on it.

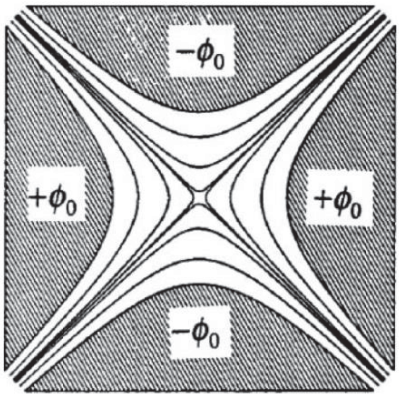

Figure 1.6. Quadrupole with hyperbolic rods and applied potential
Quadrupole with hyperbolic rods and applied potential. Reproduced from Elemental analysis with quadrupole mass filters operated in higher stability regions with permission of The Royal Society of Chemistry.

Ions travel down the quadrupole between the rods. By controlling the ratio between RF voltage and DC voltage, ions with certain m/z have stable trajectories under this condition, i.e. do not collide with the rods, and they can reach the detector. In Figure 1.6, ϕ_0 contains both DC and RF voltages, which can be denoted in Eq (1-10), where U is a DC voltage applied pole to ground, and V_{RF} is a zero to peak alternating voltage applied pole to ground, Ω is the angular frequency of the RF voltage.

$$\phi_0 = U - V_{RF} \cos\Omega t \tag{1-10}$$

Therefore, the potential at any given point between the rods can be depicted in Eq (1-11), where (0,0) is defined as the center of the four rods, x and y are Cartesian co-ordinates, r_0 is the nearest distance from the center to the rod surface.

$$\phi(x,y,t) = \left(\frac{x^2 - y^2}{r_0^2}\right) \times (U - V_{RF} \cos\Omega t) \tag{1-11}$$

When an ion with charge ze and mass m travels through the rods, its motion and

trajectory are determined by the electrical force from the rods. Conceptually the trajectory can be denoted as a certain function according to Newton's Law; however, it is quite difficult to solve the equation analytically. Instead of pursuing the analytical solution, a stability diagram can be used to predict whether the ion with m/z will collide with the rods or not. The first stability region of a linear quadrupole is reprinted below in Figure 1.7, where a and q are functions related to the m/z of an ion, defined in Eq (1-12).

$$a = \frac{8zeU}{m\Omega^2 r^2_0} \quad , \quad q = \frac{4zeV_{RF}}{m\Omega^2 r^2_0} \tag{1-12}$$

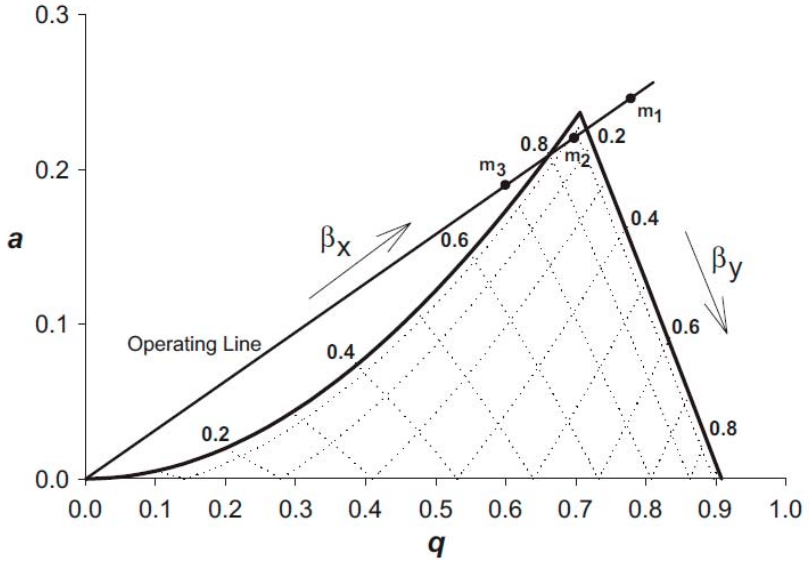

Figure 1.7. The first stability region of quadrupole with an operating line.[61] Reproduced from Elemental analysis with quadrupole mass filters operated in higher stability regions with permission of The Royal Society of Chemistry.

Because the distance and shape of quadrupoles are pre-set during manufacture, when changing U and V, an ion with certain m/z can stay in the stable region shown in the Figure 1.7: this then means that the ion will make it through to the detector. If the

ion is excluded from the stability region because of the changing voltage, it will collide on the quadrupole rods in terms of trajectory.

1.2.2.3. 3D ion trap [62,63]

The 3D ion trap is also known as the Paul trap, to honor its inventor Wolfgang Paul who shared the Nobel Prize in Physics in 1989 for this invention. The Paul trap also makes use of a quadrupole field, which consists of a ring electrode and two end cap electrodes. All of the electrodes have a hyperbolic inner surface (Figure 1.8). A quasi-quadrupole field is produced in the ion trap when the two end cap electrodes are grounded and ϕ_0 (Eq (1-10)) containing both the DC and RF components is applied to the ring electrode.

In the same way, the trajectories of ions in the Paul trap are solvable but it is quite difficult to reach an analytical solution. Employing a similar idea, a stability diagram (Figure 1.9) can be constructed to determine whether an ion with m/z is 'stable' in the trap or 'not stable' and colliding with the electrodes. In the Figure 1.9, the parameters a_z and q_z are defined in Eq (1-13), which have the forms as mentioned in the quadrupole mass analyzer stability diagram. Note that r and z are the cylindrical coordinates, and (0,0) is the center of the ion trap, i.e. the two asymptotes intersection, r_0 is the shortest distance between (0,0) and the ring electrode surface, and z_0 is the shortest distance from the origin to the cap electrode surface.

$$a_z = -\frac{16zeU}{m\Omega^2(r_0^2 + 2z_0^2)} \qquad q_z = \frac{8zeV_{RF}}{m\Omega^2(r_0^2 + 2z_0^2)} \quad , \tag{1-13}$$

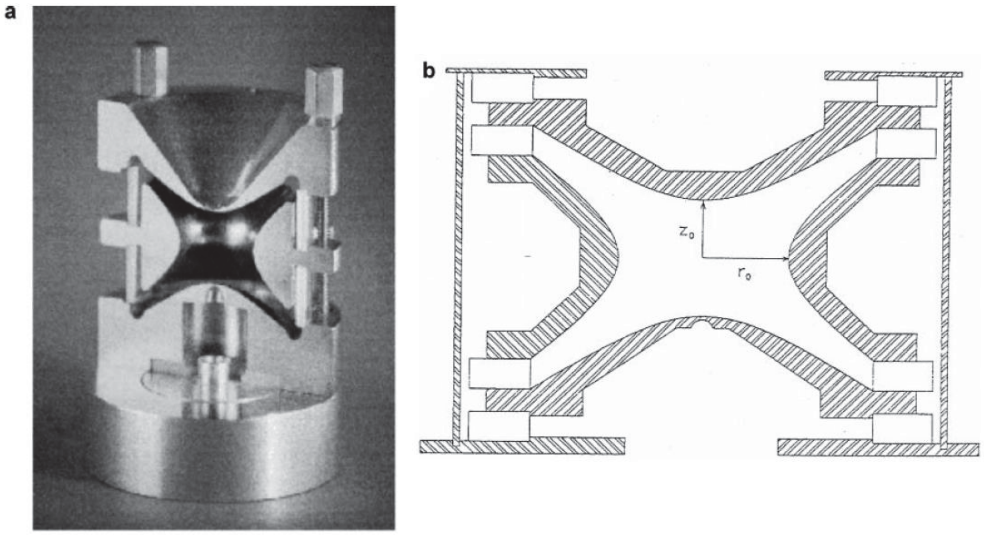

Figure 1.8. A typical 3D ion trap mass analyzer

A. A picture of a 3D ion trap mass analyzer; B. The diagrammatic figure of the 3D ion trap with the distance defined, where the origin of the coordinates is the center of the ion trap, i.e. the intersection of two asymptotes. Reprinted from Quadrupole ion traps. Copyright © 2009 Wiley Periodicals, Inc.

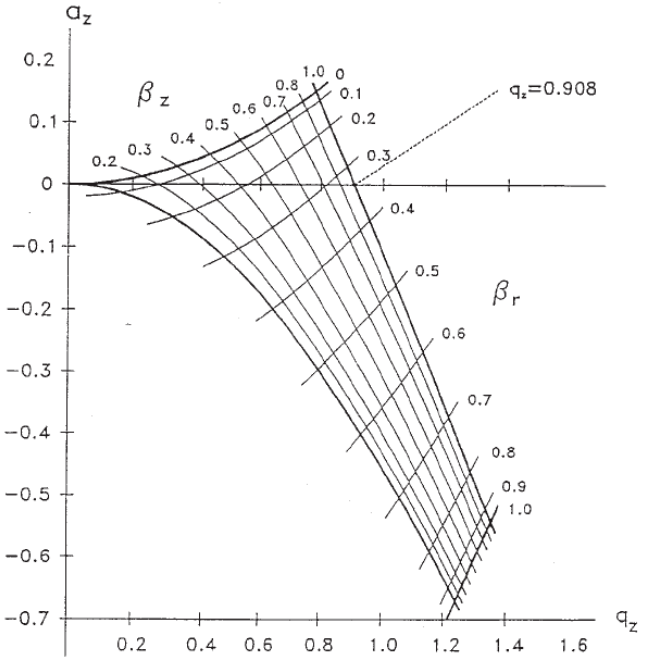

Figure 1.9. A stability diagram of the 3D ion trap

Under the applied DC and RF voltage, when an ion with m/z can stay in the stability region, it is considered 'stable'. Otherwise, it will strike the electrodes, and be considered 'unstable'. [63] Copyright © 1997 John Wiley & Sons, Ltd.

If the calculated (a_z, q_z) under given conditions for an ion is within this stability region, it will be confined in the 3D ion trap without colliding with the electrodes. Also, similar to the quadrupole field, varying U and V can include or exclude an ion from the stability region.

1.2.2.4. Other mass analyzers

Besides TOF, quadrupole and 3D ion trap, there are other types of mass analyzers, employing different principles to discriminate the ions based on their m/z ratios electrically or magnetically. To learn more about other types of mass analyzers, readers are referred to the MS textbooks. [64,65]

1.2.3. Combination of mass analyzers - MS^n

The aforementioned mass analyzers can be coupled together to perform multi-stage m/z selections. In between the multiple stages, fragmentations of ions occur. This process is called tandem MS or MS^n.

MS^n can be performed alone by 3D ion trap with the ejection of unwanted ions and fragmentation of target ions occurring in the trap. It can also be carried out by the combination of mass analyzers, such as triple quadrupole (QQQ) or quadrupole-TOF (q-TOF). If a combination of mass analyzers is used, there is usually a collision cell in between the two mass analyzers, where ions are either struck by gas molecules, or undergo other forms of fragmentation. In the case of QQQ, the first and third quadrupoles are mass analyzers while the second one is a collision cell. In q-TOF, there is a hexapole or octapole between the quadrupole and TOF, acting as the collision cell.

In MS^n, the ions selected by the first-step mass analyzer are called parent ions and those detected by the second-step mass analyzer are called daughter ions. By choosing the proper parent ion – daughter ion pair, the specificity or selectivity of an MS method towards a particular compound can be enhanced. The sensitivity is also boosted because the mass analyzers (except for TOF mass analyzers) spend more time on the specific ions than scanning the whole mass range, i.e. there is a better duty cycle for the mass analyzers. Moreover, since fragmentation is induced in different ways, structural information of compounds can be provided. [64]

1.3. Coupling CE and MS via ESI

It is intriguing to couple CE and MS through ESI to expand the applications for these two analytical techniques. CE has been proven to be superior to other separation techniques in terms of resolution but the short optical pathways associated with the narrow diameters of the capillary impose relatively low sensitivity; ESI-MS offers low limits of detection and provides structural information of analytes but somehow lacks resolving power especially for isobaric compounds and complex matrices. The combination of CE-ESI-MS was first demonstrated by Smith and co-workers in 1987. [66] In the set-up, a metal sheath around the end of capillary replaced the terminal electrode for CE and gaseous ions were generated there for MS analysis. Ever since then, there have been enormous efforts spent in the development of CE-ESI-MS interfaces. [67]

A few general concerns arise from the nature of CE and online ESI-MS detection. Both CE and ESI require stable electrical contact at the capillary terminus,

which means interruptions between the two processes are not tolerated. The BGE used in CE needs to be ESI compatible. Also, a discrepancy usually exists between the flow rate required by ESI and the bulk flow rate of a CE separation.

From the CE side, the volumetric flow rate can be varied by multiple factors, including capillary diameter, length, inner surface pretreatment, separation voltage and BGE composition. For ESI, the optimal flow rate can be determined also by multiple factors, including the geometry of the emitter, spray voltage and compositions of solution. The common flow rates from CE are no more than a few hundred nanoliters per minute but typical ESI usually requires 1-20 microliters per minute.

1.3.1. Sheath-flow or make-up liquid assisted interfaces [68]

In terms of current commercial availability, sheath-flow assisted CE-ESI-MS interfaces have been popular. The sheath-flow or make-up liquid is an additional liquid stream added at the outlet of the capillary, mixing with the CE flow at the exit. It helps to stabilize the current and acts as the electrical contact for both CE and ESI. Also, the composition of the sheath-flow can be different from BGE, thus it can adjust the compatibility between the CE effluent and the proper solution required by the ESI process. Moreover, the sheath-flow can also make up the flow rate mismatch between CE and ESI.

The sheath-flow assisted CE-ESI-MS interfaces provide good coupling solutions for the combination between CE and MS, especially during the earlier days of CE-MS development when LC-MS had already been prevalent and CE-MS interfaces could

be applied to similar set-ups. The major problem of this development is that there has always been dilution associated with the addition of sheath-flow. The dilution factor ranges from 1/2 to 1/250, depending on the CE volumetric flow rate and the emitter arrangements (Figure 1.10). Another concern is that with the junction-at-tip design (Figure 1.10 B and C), a dead volume could be introduced and cause band-broadening at the end of separation.

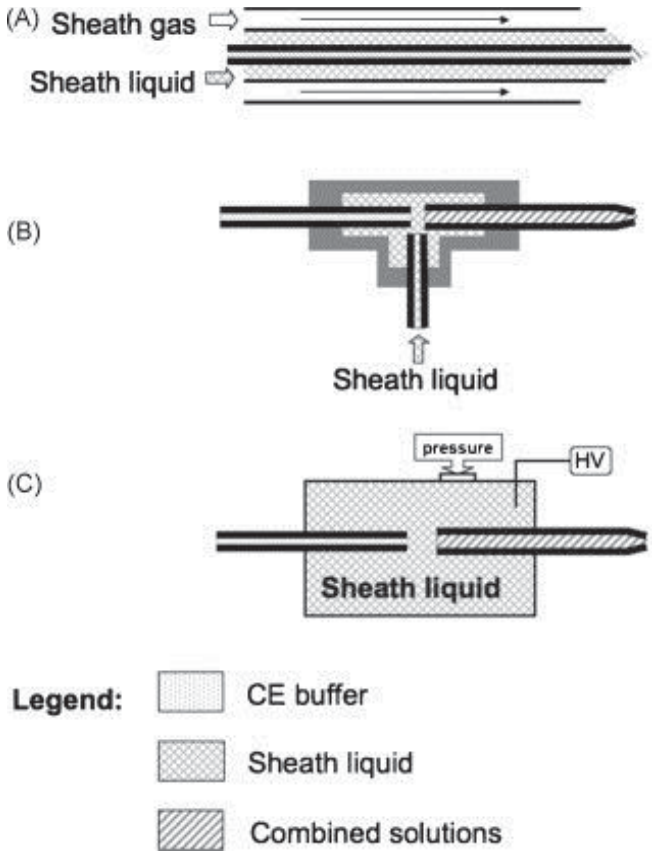

Figure 1.10. Common sheath-flow interface arrangements.
(A) Coaxial sheath-flow interface with sheath gas; (B) liquid junction-at-tip interface; (C) pressurized liquid junction-at-tip interface. Reprinted from Analytica Chimica Acta, Vol 627, E. Jane Maxwell, David D.Y. Chen, Twenty years of interface development for capillary electrophoresis–electrospray ionization–mass spectrometry, Pages 25-33, Copyright (2008), with permission from Elsevier.

1.3.2. Sheath-less interfaces [67]

To minimize the dilution effect from the sheath-flow, sheath-less CE-ESI-MS

interfaces have been proposed. To maintain a stable electrospray under low flow rate, i.e. to accommodate the flow rates provided by the CE EOF, the emitter tip size has to be reduced. One common way to achieve this is to pull out the silica capillary to a thin tip from the end of the separation capillary, which is already quite small in size. Also, some sheath-less interfaces employ metal pieces in the capillary. The manufacturing methods have been reviewed adequately. As in most analytical methods, there is a trade-off between sensitivity (no dilution factor) and robustness (the easy handling of the interfaces).

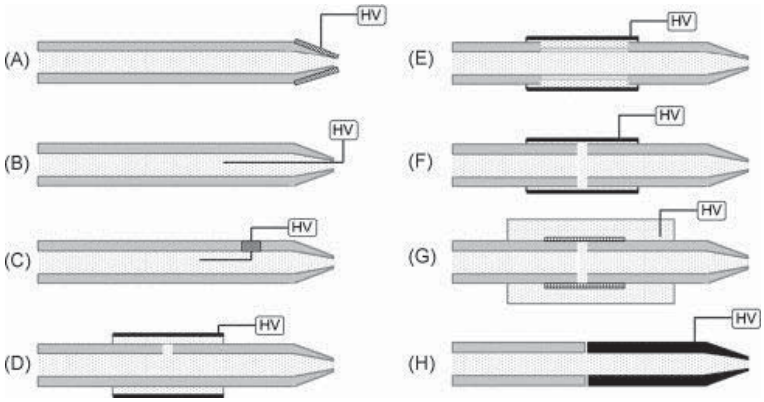

Figure 1.11. Methods for maintaining electrical contact in sheathless interfaces. (A) Emitter tip coated with conductive coatings, (B) tip with inserted wire, (C) tip with additional wire inserted through a hole, (D) using a metal sheath, (E) using a metal sleeve, (F) junction with metal sleeve, (G) microdialysis junction, and (H) junction with conductive emitter tip. Reprinted from Analytica Chimica Acta, Vol 627, E. Jane Maxwell, David D.Y. Chen, Twenty years of interface development for capillary electrophoresis–electrospray ionization–mass spectrometry, Pages 25-33, Copyright (2008), with permission from Elsevier.

To maintain a good electrical contact for both CE and ESI with sheath-less CE-ESI-MS interfaces is not easy. In the absence of a CE outlet vial, the capillary terminus has to serve as the electrode as well as the spray emitter electrode to close

the electrospray circuit. With sheath-flow assisted interfaces, the problem is not difficult to solve: the metal piece at the end can provide the electrical contact. Neither are with the sheath-less interfaces employing metal pieces. For the commonly used silica or glass based interfaces, many ways of enabling the electrical contact have been proposed, summarized in Figure 1.11.

1.3.3. Interface used in thesis study

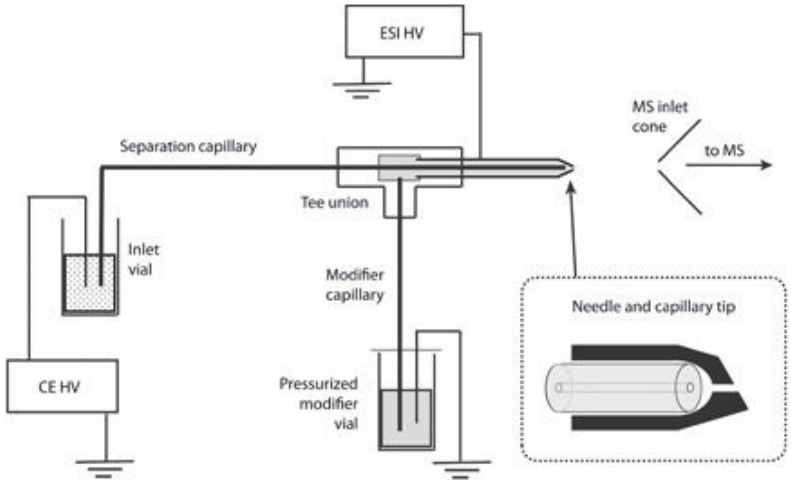

Figure 1.12. CE-ESI-MS interface used in thesis study

The interface used in the thesis study is a stainless steel beveled needle. The inner diameter fits the outer diameter of the separation capillary. When the capillary is inserted to the end of the needle, a tiny space is left at the end, called flow-through microvial. The make-up flow is supplied through another capillary connected to the needle via a stainless steel tee unit. The make-up flow and CE effluent mix at the flow-through microvial and move to the outer surface of the needle, where the electrospray occurs. Copyright © 2010 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

In the following chapters, a CE-ESI-MS interface with a flow-through microvial is used for biological sample studies. It is a stainless steel beveled emitter, whose diagrammatic set-up is shown in Figure 1.12, developed by Maxwell and co-workers, employing the junction-at-tip concept. [69] In short, the inside of the emitter

accommodates the separation capillary and when the separation capillary is inserted and pushed to the end of the inside of the emitter, there will be a tiny space left. The tiny space is called the flow-through microvial, which forms the outlet buffer vial for CE. Another capillary, the modifier capillary, supplies the make-up flow via a tee union to adjust the composition of the effluent as well as increase the total volumetric flow rate to a certain range to maintain a stable electrospray. The CE eluent and make-up flow mix in the flow-through microvial and move to the outer surface of the emitter and form the electrospray. Since the emitter is beveled, the strongest electric field only exists at the sharpest point of the tip, as does the Taylor cone.

1.4.Research Objectives

1.4.1. Optimization of cIEF-ESI-MS strategies using the flow-through microvial interface

The feasibility of online cIEF-ESI-MS employing the flow-through microvial CE-MS interface has been reported. [28] Further improvement on the reproducibility and separation efficiency is still needed before moving into the applications of real samples. Also, a deeper understanding of the cIEF focusing and mobilization processes is necessary for future experiment designs and applications.

Most of the cIEF experiments are carried out in capillaries with inner surface modifications. The interactions between the surface modification, carrier ampholytes, and focusing medium are important issues to be investigated. The understanding and controllability of the interactions are essential for improving the reproducibility of this technique. Chapter 4 is devoted to discussing the interactions among different

capillary coatings (including hydroxypropylcellulose, polyvinylalcohol, dimethylpolysiloxane, fluorocarbon, and bare fused silica), four designer-brand broad range carrier ampholytes (Fluka, Pharmalytes, Servalyt, and Bio-lyte) and three focusing media (water, 30% glycerol, and 50% Beckman cIEF polymer gel). The combinations with small consistent forward EOF and/or almost no EOF are listed and guidelines for achieving good focusing and successful chemical mobilization are provided.

Although there have been computer simulations and theoretical predictions on the cIEF process, no experimental results have shown how the carrier ampholytes form the pH gradient and how they distribute across the capillary. Chapter 5 reports the direct observation on the shape of focused ampholyte bands in the cIEF process by online cIEF-ESI-MS. Those directly detected bands also show the potential to enable a more precise pI determination of unknown amphoteric molecules.

1.4.2. Applications of the flow-through microvial interface in complex samples

As reviewed in Chapter 2 and Chapter 3, CE-ESI-MS has been applied to different complex samples. The feasibility of this flow-through microvial interface has thus far only been demonstrated for the analysis of pre-mixed standards. In Chapter 6, using this interface, CZE-ESI-MS has been used to monitor metabolite in human embryonic stem cell (hESCs) culture.

1.4.3. Incorporation of atmospheric ion lens into the CE-ESI-MS interface for higher sensitivity

An atmospheric ion lens was proposed and applied to an ESI ion source and showed extended stable operational region for flow rates. [70,71] Employing the same idea, Chapter 7 demonstrates that incorporating the atmospheric ion lens into the flow-through microvial CE-ESI-MS interface improves the electrospray ionization and sampling efficiency in the reduced flow rate region, which increases the sensitivity of the interface. A mixture of amino acids was tested to display the increased signal-to-noise ratio. The atmospheric ion lens also gives more flexibility when choosing the EOF and chemical modifier flow rates.

In Chapter 7, computer simulations and calculations also demonstrate the electric field distributions with and without the atmospheric ion lens, offering theoretical explanations for the enhanced detection sensitivity.

Chapter 2. Review of applications of capillary electrophoresis in characterizing recombinant protein therapeutics

2.1. Introduction

In 1982, recombinant human insulin was approved by the FDA and became the first commercially available recombinant protein therapeutic. [72] In the 31 years since then there has been a remarkable increase in the therapeutic applications of recombinant proteins. Currently, more than 200 protein-based drugs are commercially available and 58 biopharmaceuticals gained approval from January 2006 to June 2010. [73] Pharmacologically, therapeutic proteins can be categorized in one of five ways based on their function. They can be proteins that augment existing pathways, provide novel function or activity, interfere with a molecule or organism, deliver other proteins or compounds, or act as replacements for deficient or abnormal proteins. [74] Complementing traditional small-molecule drugs, therapeutic proteins hold a great promise for human therapy to treat hematological and solid tumors, autoimmune, inflammatory, infectious, and cardiovascular diseases. [75]

Protein therapeutics produced by recombinant technology are almost inevitably heterogeneous due to the biotechnological mode of manufacturing. In addition, potential chemical and physical changes may be introduced from the raw materials, the host cells, and the manufacturing and storage processes. [76] The chemical changes are covalent changes, e.g., amino acid sequence variants are often produced in the biosynthesis. Sequence variants may arise from genomic mutations, e.g. nucleotide starvation during the clone selection, or amino acid misincorporation during protein

translation. Protein post-translational modification (PTMs) is another type of chemical change, which often includes glycosylation, phosphorylation, oxidation, sulphation, lipidation, disulphide bond formation, deamidation, fragmentation, hydrolysis, and isomerization (transpeptidation). [77,78] Other types of post-production covalent modifications can also occur. [79] Different from chemical changes, physical changes are non-covalent, such as aggregation and denaturation.

This inherent heterogeneity can alter the protein activity and/or influence the immunogenicity. The chemical and physical changes may lead to small perturbations in the protein higher-order structure, which may cause the drug to lose its efficacy or to malfunction, although sometimes the consequences in terms of side effect are fairly benign. [77] To ensure the safety, quality, integrity and efficacy of a therapeutic protein which is usually of extreme structural complexity, it is critical to characterize and monitor the product-related variants throughout the discovery and quality control processes. A wide range of analytical techniques have been applied. [76]

Capillary electrophoresis (CE) is an effective technique to characterize proteins. It is beneficial with respect to its simple instrumentation, superior separation efficiency, small sample consumption, and short analysis time. The versatility of CE is attributed to the different separation modes available on the same hardware, and the feasibility of coupling it to various detection methods, such as laser-induced fluorescence (LIF) and mass spectrometry (MS). The major drawback of CE analysis compared to liquid chromatography (LC) is the limited concentration sensitivity. However, in the context of therapeutic protein characterization, high-concentration

samples are often easily available. Thus, as an orthogonal and complementary analytical technology, CE has proven its effectiveness to characterize therapeutic proteins and the FDA has fully accepted CE in process development, characterization and QC release.

In this review article, the application of CE to the analysis of recombinant protein therapeutics from 2000 to 2013 is summarized. The reported studies are categorized according to separation mode, including capillary isoelectric focusing (cIEF), sodium dodecyl sulfate capillary gel electrophoresis (CE-SDS), capillary zone electrophoresis (CZE), and capillary electrophoresis-mass spectrometry (CE-MS). The principles are briefly reviewed and technical concerns are discussed. There have been adequate reviews on other characterization methods. [77,80] Also, CE for protein analysis has been reviewed by other authors. [81,82]

2.2.Capillary isoelectric focusing

2.2.1. Principles

cIEF separates molecules based on their isoelectric points (pIs) in a capillary format. The experimental set-up usually includes a basic catholyte at the cathode and an acidic anolyte at the anode. A mixture of the protein to be analyzed and carrier ampholytes fills up the majority of the separation capillary. A protein becomes positively charged when its pI value is higher than that of the local pH, and negatively charged in the opposite situation. When an electric field is applied to the capillary, the protein will migrate towards the oppositely charged electrode. As the protein migrates along the pH gradient, the charge on the protein will decrease to zero when it reaches

the region where the local pH is the same as its own pI. At the pI, the electrophoretic mobility of the particle should be zero, so that proteins, which fill up the whole capillary at the beginning, are focused into sharp bands according to their pIs in a pH gradient along the capillary. [19]

2.2.2. Applications

cIEF has become an often used separation technique for protein charge variants, which could arise from PTMs, such as deamidation, C-terminal lysine processing, cysteinylation and glycation. [78] The characterization of charge heterogeneity may also lead to the discovery of sequence variants. [83] cIEF can also be used to identify impurities from the therapeutic proteins. The recent applications of cIEF in protein therapeutic analysis are summarized in Table 2.1.

2.2.3. Technical concerns

2.2.3.1.Imaging and two-step cIEF

The focused protein bands can be detected in one of two ways: whole column imaging, or single-point detection with mobilization, also known as two-step cIEF. In this review, concurrent pressure and EOF mobilization during two-step cIEF is not discussed; however, relevant information can be found in other review articles.[17] Whole column imaging cIEF (icIEF) enables real-time monitoring of the focusing process. The most popular commercial instrument iCE 280 [84] features a 5-cm long capillary, so the focusing can be accomplished in a short time. The short capillary length also means that a strong electric field can be achieved by applying a relatively low separation voltage. The trade-off here is the resolution: the two-step cIEF method

with much longer capillaries offers better resolution. Coupling novel detection methods, such as LIF or MS, is more straightforward with the two-step cIEF.

2.2.3.2.Protein solubility enhancement

During a cIEF process, when the current drops to its minimum, the proteins are focused into bands in accordance to their pI. At this time, they are neither positively nor negatively charged; they are also at relatively high concentration, which could cause the proteins to precipitate. Protein precipitation could lead to partial clogging of the capillary, chemical mobilization failure and/or irreproducibility of the analysis. To enhance protein solubility, urea, at various concentrations, is usually added to the sample mixture. [22,29,84-90]

2.2.3.3.Sacrificing agent

The anodic and cathodic drifts caused by the bidirectional isotachophoresis could induce a temporal pH gradient instability in IEF and could lead to sample loss. [91] The use of concentrated anolyte and catholyte could reduce the rate at which extreme pI carrier ampholytes and sample lose, but is unable to completely eliminate the situation. Adding sacrificial ampholytes to the sample mixture can sufficiently save the interested part of focused zones. Mack *et al.* [29] carried out a systematic study on the prevention of the loss of carrier ampholytes and sample components during focusing. Arginine (pI 10.7) and iminodiacetic acid (pI 2.2) were chosen to be the ideal sacrificing agents (also called blockers or spacers) and the concentrations of these compounds are optimized. Many analyses on therapeutic proteins have incorporated sacrificial compounds [22,86-88] and arginine and iminodiacetic acid do not have to be

present at the same time.

Adding sacrificial ampholytes in two-step cIEF can also ensure the observation of analyte zones during the mobilization step by acting as a spacer to confine the analytes in the front of the detection window, so that it only migrates through the detection window after the mobilization process is initiated. [29] Without the sacrificing agents, peaks that are artifacts of analytes band migrating back and forth around the detection window can be observed, causing confusions when the results are interpreted.

2.2.3.4.Detection

Almost all current cIEF analyses use UV absorption detection at 280 nm. In iCE 280, the proteins are focused and the whole 5-cm column is imaged by a CCD camera. In the two-step cIEF experiments, the protein train passes the detection window sequentially, and the detection aperture size has an influence on the resolution [29].

UV detection is sufficient when the protein concentration is adequate. Two ways can help to push the detection limit lower. Michels *et al.* [22] applied a multiplex based immunoassay to icIEF for the charge heterogeneity characterization of the in-house manufactured monoclonal antibodies (mAbs) (Figure 2.1). After focusing, the IgG mAbs were immobilized to the capillary wall by UV light and probed using a horseradish peroxidase conjugated secondary anti-IgG. The bound anti-IgG, after washing, were detected by chemiluminescence with a peroxidase reactive substrate. This immunoassay had a LOD of 2 ng/mL for the charge variants.

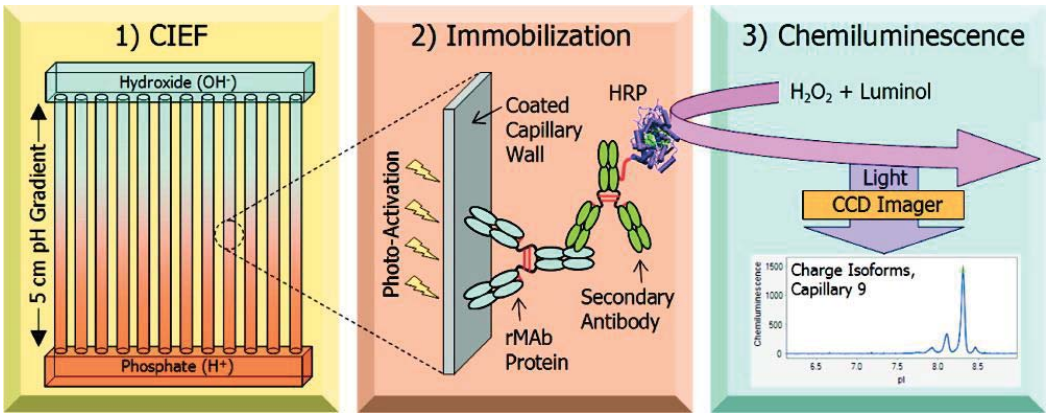

Figure 2.1. Charge heterogeneity of monoclonal antibodies by multiplexed icIEF immunoassay with chemiluminescence detection. Reprinted with permission from [21]. Copyright (2012) American Chemical Society

Another way to increase the sensitivity is to couple cIEF with MS. [17] The hyphenation between cIEF and MS provides the separation based on pI and mass-to-charge ratio, which could be equivalent to a simplified version of two-dimensional gel electrophoresis. Moreover, with MS/MS, protein structural information can be provided without further separation procedure. Zhu *et al.* [92] analyzed host cell proteins for a mAb via cIEF-MS. With the in-house made electrospray interface, the LOD can be 70 nM for a myoglobin peptide.

2.2.3.5.Capillary wall conditioning

Arguably cIEF is the analytical technique with the highest resolution, reproducibility has always been a concern. Even using commercialized protocols, consecutive runs can cause significant capillary degradation. The degradation is often resulted from unstable capillary wall coating, protein adsorption, and carrier ampholytes dynamic interaction with the capillary wall. Much effort has been made

for removing the absorbed proteins, including rinsing with hydrochloric acid and urea solutions. [93] Recent articles used urea to rinse between runs to maintain performance. [29,86-88] Bonn *et al.* [93] implemented a revival solution wash in combination with a PVA-coated capillary and increased number of reproducible runs from 4–5 injections to 100+ injections per capillary.

Table 2.1. Protein therapeutics analysis by CIEF in 2000-2012

Sample	Mode	Capillary	Ampholytes	Catholyte	Anolyte	Sacrifizer	Author
IgG1 mAbs	iCIEF	iCE280	Pharmalyte				Yin[94]
mAb1	iCIEF	iCE280	Pharmalyte 3-10 spiked with 8-10.5	0.1 M NaOH in 0.1% MC	0.08 M H_3PO_4 in 0.1% MC		Anderson[95]
all other mAbs			Biolyte 3-10 spiked with 8-10				
EPO			Pharmalyte 4-6.5, 5-8 and 8-10.5 1:1:1				
Fc-fusion proteins			Servalyt 3-10				
Fc-fusion sialidase A treated samples			Pharmalyte 3-10				
in-house manufactured IgG1 mAbs	iCIEF immunoassay	proprietary photoreactive layer coated	Pharmalytes 5-8 and 8-10.5 3:7	0.1 M NaOH in 0.1% MC	0.08 M H_3PO_4 in 0.1% MC	2.5 mM arginine	Michels[22]
anti-α1-antitrypsin mouse mAb	iCIEF	iCE280	pH 5-8 GE healthcare ampholytes	0.1 M NaOH in 0.1% MC	0.08 M H_3PO_4 in 0.1% MC		Salas-Solano[85]
ch14.18	iCIEF	iCE280	Pharmalyte 3-10 spiked with 8-10.5	0.1 M NaOH	0.08 M H_3PO_4		Soman[96]
in-house manufactured mAbs	iCIEF	iCE280	Pharmalyte 3-10 spiked with 8-10.5				Lehermayr[97]
in-house manufactured mAbs	two-step	PVA-coated	Pharmalyte 3-10 spiked with 8-10.5	0.3 M NaOH	0.2 M H_3PO_4	50 mM arginine	Lin[86]
in-house manufactured mAbs	iCIEF	iCE280	Pharmalyte 3-10 spiked with 8-10.5				Ren[83]
in-house manufactured IgG1 mAbs	two-step	neutral coated	Pharmalytes 5-8	0.3 M NaOH	0.2 M H_3PO_4	0.5 M arginine and 0.2 M IDA	Salas-Solano[87]

Table 2.1. Protein therapeutics analysis by CIEF in 2000-2012

Sample	Mode	Capillary	Ampholytes	Catholyte	Anolyte	Sacrifizer	Author
IgG1 mAbs	iCIEF	iCE280	Pharmalyte				Yin[94]
mAb1	iCIEF	iCE280	Pharmalyte 3-10 spiked with 8-10.5	0.1 M NaOH in 0.1% MC	0.08 M H_3PO_4 in 0.1% MC		Anderson[95]
all other mAbs			Biolyte 3-10 spiked with 8-10				
EPO			Pharmalyte 4-6.5, 5-8 and 8-10.5 1:1:1				
Fc-fusion proteins			Servalyt 3-10				
Fc-fusion sialidase A treated samples			Pharmalyte 3-10				
in-house manufactured IgG1 mAbs	iCIEF immunoassay	proprietary photoreactive layer coated	Pharmalytes 5-8 and 8-10.5 3:7	0.1 M NaOH in 0.1% MC	0.08 M H_3PO_4 in 0.1% MC	2.5 mM arginine	Michels[22]
anti-α1-antitrypsin mouse mAb	iCIEF	iCE280	pH 5-8 GE healthcare ampholytes	0.1 M NaOH in 0.1% MC	0.08 M H_3PO_4 in 0.1% MC		Salas-Solano[85]
ch14.18	iCIEF	iCE280	Pharmalyte 3-10 spiked with 8-10.5	0.1 M NaOH	0.08 M H_3PO_4		Soman[96]
in-house manufactured mAbs	iCIEF	iCE280	Pharmalyte 3-10 spiked with 8-10.5				Lehermayr[97]
in-house manufactured mAbs	two-step	PVA-coated	Pharmalyte 3-10 spiked with 8-10.5	0.3 M NaOH	0.2 M H_3PO_4	50 mM arginine	Lin[86]
in-house manufactured mAbs	iCIEF	iCE280	Pharmalyte 3-10 spiked with 8-10.5				Ren[83]
in-house manufactured IgG1 mAbs	two-step	neutral coated	Pharmalytes 5-8	0.3 M NaOH	0.2 M H_3PO_4	0.5 M arginine and 0.2 M IDA	Salas-Solano[87]

Sample	Mode	Capillary	Ampholytes	Catholyte	Anolyte	Sacrifizer	Author
in-house manufactured mAbs	iCIEF	iCE280	pH 3-10 spiked with 6.7-7.7	0.1 M NaOH in 0.1% MC	0.08 M H_3PO_4 in 0.1% MC		Zhang[98]
Deglycosylated gemtuzumab ozogamicin	two-step	neutral coated or dimethylpolysiloxane-coated	Pharmalyte 3-10	0.3 M NaOH	0.2 M H_3PO_4	0.5 M arginine and 0.2 M IDA	Maeda[88]
in-house manufactured IgG2 mAbs	two-step		Pharmalyte 3−10				Meert[99]
three proprietary mAbs	two-step	neutral coated	Pharmalyte 3-10	0.3 M NaOH	0.2 M H_3PO_4	1.7 mM IDA and 40 mM arginine	Mack[29]
mAbs provided by Pfizer or AmerisourceBergen	iCIEF	iCE280	Ampholine 3-10	NaOH	0.08 M H_3PO_4 in 0.1% MC		He[100]
THIOMABs	iCIEF	iCE280	Pharmalyte 3-10 spiked with 8-10.5	0.1 M NaOH in 0.1% MC	0.08 M H_3PO_4 in 0.1% MC		Chen[101]
IgG1 mAb digested by Carboxypeptidase B	iCIEF	iCE280	Pharmalyte 3-10 spiked with 8-10.5	0.4 M NaOH in 0.1% MC	0.1 M H_3PO_4 in 0.1% MC		Apostol[102]
in-house manufactured mAbs	two-step	neutral coated					Dick[103]
rhEPO	iCIEF	iCE280	Fluka Ampholytes 3-10	0.1 M NaOH in 1% (w/v) polyvinylpyrrolidone	0.1 M H_3PO_4 in 1% (w/v) polyvinylpyrrolidone		Dou[89]
hirudin, erythropoietin, and bevacizumab	iCIEF	MCE-2010 linear PA coated, 50 μm× 110 μm×25 mm	BioChemika 3-10 spiked with Pharmalyte 2.5-5	0.02 M NaOH	0.01 M H_3PO_4		Vlčková[104]

Sample	Mode	Capillary	Ampholytes	Catholyte	Anolyte	Sacrifizer	Author
in-house manufactured recombinant proteins	iCIEF	iCE280	Pharmalyte 3-10 spiked with 8-10.5 or 5-8	0.1 M NaOH	0.08 M H_3PO_4		Sosic[90]
in-house manufactured mAbs	iCIEF	iCE280	Pharmalytes	0.1 M NaOH in 0.1% MC	0.08 M H_3PO_4 in 0.1% MC		Quan[105]
a glycosylated, a non-glycosylated and a PEGylated protein model	iCIEF	iCE280	Pharmalyte 3-10 spiked with 8-10.5	0.1 M NaOH	0.08 M H_3PO_4		Li[84]
MU-B3	iCIEF	iCE280	Pharmalyte 3-10 and 5-8 1:3	0.1 M NaOH	0.1 M H_3PO_4		Janini[106]

iCE 280, 50 mm long 100 μm id; fluorocarbon coated

MC methylcellulose

IDA iminodiacetic acid

Table 2.2. Protein therapeutics analysis by CE-SDS in 2000-2012

Sample	Mode	Protein treatment	Separation	Buffer	Detection	Reference
IgG1 mAbs	reduced	labeled with FQ dye	-15 kV		LIF 488 nm excitation, 600±15 nm emission	Yin[94]
carbonic anhydrase I, myoglobin, ovalbumin, and BSA			50 μm×33(24.5) cm; -16.5 kV	Beckman Coulter SDS-MW gel buffer	UV 220 nm	Cianciulli[116]
anti-α_1-antitrypsin			50 μm×50(45) cm; +20 kV	Tris and borate buffer with various SDS concentrations	UV 200 nm	Cooper [107]
in-house produced IgG1 and IgG4 mAbs		buffer exchanged and labeled with FQ dye	50 μm×31.2(20) cm; -15 kV	Beckman Coulter SDS-MW gel buffer	LIF 488 nm excitation, 600 ± 20 nm emission	Michels [117]
RTA conjugated mAbs	reduced	conjugated to RTA after SPDP modification	50 μm× 24(19.5) cm; -15 kV	Biorad CE-SDS run buffer	UV 220 nm	Na[108]
in-house produced vaccines	reduced CE-SDS western blotting		100 μm× 5 cm; 250 V	Protein Simple separation matrix and stacking matrix	proteins immobilized by UV light, probed by a secondary antibody and detected by chemiluminescence	Rustandi [110]
bovine serum IgG, and mouse serum IgG	non-reduced and reduced	labeled with flurofore Chromeo P503	QIAxcel multi-capillary electrophoresis instrument; 6 kV	60 mM TRIS–borate containing 0.1 % SDS (pH 8.45)	LED induced fluorescent detection	Szekrényes [118]

Sample	Mode	Protein treatment	Separation	Buffer	Detection	Reference
in-house produced mAbs	reduced and non-reduced	Lys-C and trypsin digestion for nonreduced	50 μm×31.2(20) cm; -15 kV	Beckman Coulter SDS-MW gel buffer	UV 220 nm	Hapuarachchi [119]
in-house produced mAbs	reduced and non-reduced	buffer exchanged and labeled with FQ dye	50 μm×31.2(20) cm; -15 kV	Beckman Coulter sieving gel	LIF 488 nm excitation, 600 nm emission	Kaschak [120]
in-house produced mAbs	reduced		50 μm×31.2(20) cm; -15 kV	Beckman Coulter sieving gel	UV 220 nm	Kotia [121]
in-house produced IgG2 mAbs	reduced and non-reduced	Lys-C digested	50 μm×30.2(20.2) cm	Beckman Coulter sieving gel	UV 220 nm	Lacher [115]
in-house produced mAbs	reduced and non-reduced		50 μm×31.2(20) cm; -15 kV	Beckman Coulter sieving gel	UV 220 nm	Zhang [122]
in-house produced mAbs	reduced and non-reduced		50 μm×33(24.5) cm; -15 kV	Biorad CE-SDS run buffer	UV 220 nm	Guo [109]
				proprietary borate buffer with additives (pH 9.9) (Sebia)	UV 200 nm	McCudden [123]
in-house produced mAbs	reduced and non-reduced		50 μm×31.2(20) cm; -15 kV	Beckman Coulter sieving gel	UV 220 nm	Rustandi [124]
in-house produced mAbs	reduced	labeled with FQ dye	50 μm×31.2(20) cm; -15 kV	Beckman Coulter sieving gel	LIF 488 nm excitation, 600 ± 15 nm emission	Michels [114]
in-house produced mAbs	reduced and non-reduced	buffer exchanged using NAP-5 and labeled by 5-TAMRA.SE	50 μm×31.2(20) cm; -480 V/cm	CE-SDS polymer solution	LIF 488 nm excitation, 560 ± 20 nm emission	Salas-Solano [125]

Sample	Mode	Protein treatment	Separation	Buffer	Detection	Reference
in-house produced mAbs	reduced		50 μm i.d. × 33 cm, bubble factor 3 (extended light path); 25 kV	10% SDS, at a mass ratio of 1:1.7 (protein:SDS)	UV 220 nm	Zhang [112]
in-house produced IgG1	reduced		50 μm×38.5(30) cm; 390 V/cm	properitery buffer solution	UV 220 nm	Tous [126]

FQ dye, 3-(2-furoyl)-quinoline-2-carboxaldehyde

5-TAMRA.SE 5-carboxytetramethylrhodamine succinimidyl ester

2.4. Capillary electrophoresis - mass spectrometry

2.4.1. Principles and Applications

Coupling MS to CE significantly increased the potential applications of the CE technique by using an information rich detection method, providing information about the identity and structure of analytes. Two main types of characterization are performed when analyzing proteins by CE-MS. One is to determine the primary structures. Proteins are usually treated by enzymes to release the peptides and the peptides are separated and detected. The obtained mass-to-charge ratios are searched in the databases and the proteins in a mixture can be identified or the amino acid sequence can be elucidated. CE-MS excels at low-mass peptides and is proven to have better sequence coverage compared to LC-MS.[127,128] Another goal for characterization is to study the intact proteins for additional information. The purity of a therapeutic protein can be determined by CE-MS via the separation of impurity constituents. The isoforms of a protein, such as glycoforms, could also be characterized by CE-MS. [129-131]

Note that the coupling between CE and MS is not as straightforward as that of LC-MS. However, the feasibility has been demonstrated by the development of improved interfaces over the last 26 years. Review articles about CE-MS interfaces have been published by many authors. [67] CE-MS can also incorporate other detection methods in the same system to provide additional information. Gennaro *et al.* demonstrated a CE-LIF-MS system for the analysis of the glycans released from mAbs and obtained both qualitative (structural) and quantitative data in one run. [132]

Recent CE-MS applications are listed in Table 2.3.

2.4.2. Technical concerns

2.4.2.1.Sequence coverage

Peptide mapping is crucial for the characterization of therapeutic proteins. A protein is usually digested by enzymes like trypsin first, and followed by Matrix-assisted laser desorption ionization – time-of-flight MS (MALDI-TOF) or LC-MS analysis to elucidate the primary structure. MALDI-TOF is fast but prone to matrix effect and ion suppression. Reverse phase LC-MS provides high sequence coverage (>95%) but is poor at detecting the low-mass peptides: the retention is insufficient because of the weaker interaction between the stationary phase and the small or hydrophilic peptides when the high-salt-content digestion buffer is present. Because the separation mechanism of CE is charge-based, it tolerates salt ions in the sample and is a useful tool for the analysis of small hydrophilic molecules. Therefore, analyzing 'void' peptides that are often lost in the void peak of the chromatograms in LC-MS, by CE-MS is an attractive complement for peptide mapping. [128] Whitmore *et al*. reported a 100% sequence coverage using CE-MS for the analysis of the in-house manufactured mAbs, while the LC-MS method covered 49 out of 61 predicted peptides, missing the short peptides. The smallest eight peptides were seen only with CE-MS.[127]

2.4.2.2.CE-MS interface

The CE-MS analysis of therapeutic proteins has been carried out mainly using triple-tube interfaces. Coupling to MS limits the range of available background electrolyte (BGE) for CE analysis. In a triple-tube interface, the make-up flow can modify the chemical properties of the eluent and provide a broader range of BGE choices. The sheath gas also promotes ionization. However, the make-up flow introduces significant dilution to the analytes, which decreases the sensitivity. The sheathless interface has been used in the research community

for better sensitivity. However, only one paper reported applying the sheathless interface for peptide mapping of therapeutic proteins [127] and one other for protein isoform characterization [129].

2.4.2.3.Background electrolyte

Background electrolytes composed of formic acid and/or acetic acid are commonly used for CE-MS analysis of therapeutic proteins. The small organic acid molecules are volatile and therefore are ESI-friendly. Also, the intact protein analytes or the peptides generated from enzymatic digestions are positive ESI amenable and so acidic BGEs facilitate the ionization.

2.4.2.4.Capillary conditioning

The CE-MS analysis for therapeutic proteins so far has been carried out with acidic BGEs for which the adsorption to the bare fused silica capillary inner surface is minimal. With neutrally coated capillaries, the interaction between proteins/peptides and capillary inner surfaces is also suppressed by the coatings. Buffer flushing for a short period of time between runs is thus usually sufficient for capillary re-conditioning between runs.

Table 2.3. Protein therapeutics analysis by CE-MS in 2000-2012

Sample	Mode	Capillary	BGE	Interface	Sheath flow	MS	Sample pretreatment	Reference
rhEPO interferon-β	CZE-MS	Beckman neutral bilayer coated	2.0 M CH_3COOH 50 mM CH_3COOH	Beckman Coulter sheathless	none	TOF	Desalted	Haselberg[129]
in-house produced mAbs	CZE-MS	PVA-coated or bare fused silica	10% CH_3COOH	Agilent sheath-liquid	0.1% CH_3COOH	TOF	tryptic digestion	Whitmore[127]
		bare fused silica	10% CH_3COOH	Beckman Coulter sheathless	none	TOF		
host cell proteins of a recombinant mAb	cIEF-MS	linear polyacrylamide-coated	0.1% HCOOH; 0.3% NH_4OH; Pharmalyte 3–10; mobilized by sheath flow	in-house manufactured sheath-liquid	0.05% HCOOH in 50% methanol	Orbitrap	rhIgG depleted, magnetic microsphere-based immobilized trypsin used for digestion	Zhu[92]
in-house synthesized deamidated peptides	CZE-MS	PVA-coated	100 mM or 20 mM CH_3COOH	New Objective metal-coated nanospray		IT		Gennaro[133]
rhEPO	CZE-MS	polybrene or UltraTol dynamic Pre-Coat LN dynamically coated	1 M CH_3COOH in 20% methanol	Agilent sheath-liquid	1% CH_3COOH in 50% 2-propanol	TOF	desalted and preconcentrated	Balaguer[130]
rhEPO peptide	CZE-MS	bare fused silica	100 mM HCOOH	Agilent sheath-liquid	0.5% HCOOH in 50% 2-propanol	IT	Desalted, tryptic digestion	
in-house produced mAbs	CZE-MS	PVA-coated	25 mM HCOOH	ThermoFinnigan sheath-liquid	methanol/water/BGE (80:15:5, v/v/v)	IT	endoproteinase Lys-C digested	Gennaro[128]
rhEPO	CZE-MS	polybrene-coated	1 M CH_3COOH in 20% methanol	Agilent sheath-liquid	2-propanol:1% CH_3COOH in water (1:1)	TOF		Neusüß[131]

Sample	Mode	Capillary	BGE	Interface	Sheath flow	MS	Sample pretreatment	Reference
peptide hormones of therapeutical interest	CZE-MS	bare fused silica	50 mM CH_3COOH and 50 mM HCOOH	Agilent sheath-liquid	2-propanol-water/0.05% HCOOH (60:40 v/v)	TOF		Sanz-Nebot [134]

2.5. Capillary zone electrophoresis

2.5.1. Principles and applications

CZE is still the most often used mode of CE due to its simplicity. The separation is based on the different electrophoretic mobilities which result from the different ratios of charge and hydrodynamic radius of the analytes when they are present in an electric field.

In the manufacturing of therapeutic proteins, different PTMs can be incorporated. CZE as a charge based separation tool is usually used to confirm the identity of a therapeutic protein, detect the impurities, and characterize the charge heterogeneity. The applications of CZE are summarized in Table 2.4.

2.5.2. Technical concerns

2.5.2.1.Detection

The CZE applications so far (CZE-MS not included) rely mostly on UV detection at 200 nm or 214 nm, as shown in Table 2.4. To achieve better sensitivity, deep UV-LIF can be utilized for recombinant human erythropoietin (rhEPO) analysis. [135,136] The excitation wavelengths are below 300 nm and the emission is around 340 nm, based on the native fluorescence of rhEPO molecules.

2.5.2.2.Capillary conditioning

Different types of the capillary wall modifications (bare fused silica, neutrally coated, and positively coated) can be used for CZE applications for therapeutic proteins. For the analysis of rhEPO which is quite acidic, the rinsing sequence always consists of 0.1 M NaOH rinse followed by water and BGE. [135-137] For products like

mAbs, the conditioning process is more likely to include an additional HCl rinse step prior to NaOH wash and/or SDS wash. More details are listed in Table 4.

2.5.2.3.CZE vs. other electrophoretic separations

Since CZE has mostly been applied in charge variant monitoring for therapeutic proteins, and another commonly used assay for the charge variant is cIEF, the efficiency comparison between the two techniques is an interesting topic. Moreover, the current mainstream charge-based separation method for proteins is cIEF, so that the general applicability of a CZE method can be demonstrated by a head-to-head comparison with cIEF assay for the same sample. He *et al*. [138] reported a comparison between the icIEF and CZE analysis for the charge variants of five mAbs. The separation profiles were similar and icIEF showed higher resolution for the acidic analytes while CZE displayed better separation for the basic ones. This is only one example of the head-to-head comparison between the two techniques, and more study is required to further compare the two techniques.

The CZE method was also compared with size based separation assays. Lee *et al*. [139] carried out a study on the polyethyleneglycol(PEG)-modified granulocyte-colony stimulating factor (G-CSF). Size exclusion chromatography showed low resolution for separating mono- and di-PEG-G-CSFs. CE-SDS provided higher resolution but required a longer analysis. CZE could separate both PEG-5K and PEG-20K conjugated G-CSFs successfully within 20 mins. In this study, CZE showed better efficiency for PEGylation reaction monitoring and purity test for PEG-G-CSF. Again, more case studies are needed to support these findings.

2.6. Concluding remarks

The use of recombinant protein for human therapeutics demands effective characterizations of the drugs to ensure safety, quality, integrity and efficacy. Different CE separation modes have been used in the drug discovery and design, development, and quality control phases. cIEF, CE-SDS and CZE have been utilized routinely in the biopharmaceutical industry. CE-MS adds another dimension of separation and provides more information about the targeted protein from a single run. The versatility of CE has proven its effectiveness as a promising technique for therapeutic protein characterization. With more research ongoing in the field of therapeutic proteins, CE is playing an increasingly important role.

Table 2.4. Protein therapeutics analysis by CZE in 2000-2012

Sample	Capillary	BGE	Running condition	Sample pretreatment	Detection	Run-to-run conditioning	Reference
rituximab, trastuzumab, and ranibizumab	polyacrylamide-based hydrophilic	200 mM EACA-CH_3COOH, 30 mM CH_3COOLi, and 0.05% w/v HPMC, pH 4.8	50μm×40.2(30.2) cm; 15 kV		UV 214 nm	0.1 N HCl, H_2O, BGE	Espinosa-de la Garza [140]
infliximab and bevacizumab		150 mM EACA-CH_3COOH, 20 mM CH_3COOLi, and 0.05% m/v HPMC, pH 5.5.					
tryptic digest of a mAb	APTES coated	50% CH_3CN, 0.1% HCOOH	microchip CE channel 91 mm		TOF MS		Mellors [141]
rhEPO	UltraTrol Dynamic Pre-coat LN dynamic coated	2.0 M CH_3COOH	UV 30 kV, 75μm×70(60)cm; Flu 17.5 kV, 75μm×74(49)cm (Flu)	ultracentrifugal desalting	UV 200 nm; Flu 280-295 nm excitation, 335 nm emission	1 M NaOH, H_2O, UltraTrol dynamic pre-coat LN, BGE	de Kort [135]
interferon α-2 variant forms	polybrene coated	50 mM HCOOH in 20% CH_3OH	50μm×90(80); -30 kV	buffer exchanged	UV 200 nm	H_2O, BGE	Mark [142]
C-terminal Lys variant of TNF-α targeted monoclonal IgG1 and papain induced degradation of Her-2 targeted monoclonal IgG1	bare fused silica	20 mM CH_3COOH (pH 6.0), 0.3% PEO and 2 mM triethylenetetramine (TETA)	50μm×30.2(20)cm; 30 kV		UV 214 nm	0.1 M HCl, BGE	Shi [143]
rhEPO	bare fused silica	10 mM CH_3COONa, 7 M urea, 10 mM Tricine, 3.9 mM putrescine, and 100 mM NaCl at pH 5.50	50μm×60(50)cm; 15 kV	immuno-magnetic beads-based extraction	LIF 266 nm excitation, 340 nm emission	0.1 M NaOH, BGE	Wang [136]

Sample	Capillary	BGE	Running condition	Sample pretreatment	Detection	Run-to-run conditioning	Reference
5 commercialized mAbs	fused-silica sipper chip	0.2% HPMC and 0.1% polysorbate 20 in 50 mM CH_3COONa pH 6	5μm×12.5mm; 2000 V	derivatized by Cy5 N-hydroxysuccinimide ester	UV 214 nm	not required	Han [144]
in-house produced mAbs	bare fused silica	400 mM EACA–CH_3COOH, 0.05% m/v HPMC, 2 mM TETA, pH 5.7	40μm×30(10)cm; 30 kV		UV 214 nm	0.1 M HCl, BGE	He [138]
bevacizumab and ranibizumab	bare fused silica	PBS of 0.016 M ionic strength (pH 7.4)	50or75μm×30(20)cm; 10 kV or 6 kV		UV 212 and 254 nm	0.1 M NaOH, H_2O, BGE	Li [145]
recombinant human glycosylated interleukin-7	diaminobutane dynamically coated	25 mM $Na_2B_4O_7$ at pH 10, 12 mM diaminobutane	50μm×47(40)cm; 20 kV		UV 214 nm	H_2O, 0.1 M NaOH, H_2O, BGE	Alahmad [146]
granulocyte-colony stimulating factor	bare fused silica	100 mM phosphate buffer (pH 2.5)	75μm×32 (22) cm;10 kV		UV 214 nm	0.1 M NaOH, H_2O, BGE	Lee [139]
in-house produced mAbs	bare fused silica	600 mM EACA−CH_3COOH (pH 5.5), 0.1% HPMC	50μm×50(40)cm; 24 kV	deglycosylated	UV 214 nm	0.1 N HCl, BGE	He [147]
		600 mM EACA−CH_3COOH (pH 5.5), 0.1% HPMC, 4 M urea		redox by cysteine			
recombinant human interleukin-7	bare fused silica	25 mM citrate/TEOA at pH 2.6	75μm×57(50)cm; 15 kV		UV 214 nm	H_2O, 50 mM SDS, H_2O, BGE	Alahmad [148]
tocilizumab	carboxylated	25 mM phosphate (pH 5.8) and 0.1% SDS	50 μm×50(40) or 40(30)cm; 20 or 28 kV	PNGase F digested; reduced	UV 214 nm	1M NaCl, BGE	Taga [149]
PEGylated human parathyroid hormones	polyacrylamide-coated	100 mM phosphate buffer (pH 2.5)	50μm×24(19.5)cm; 10 kV	Lys-C digested	UV 200 nm and 280 nm	H_2O, BGE	Na [150]

Sample	Capillary	BGE	Running condition	Sample pretreatment	Detection	Run-to-run conditioning	Reference
nanoparticle–protein conjugates	bare fused silica	100 mM $Na_2B_4O_7$	75μm×65(48)cm; 30 kV	derivatized with naphthalene-2,3-dicarboxaldehyde	LIF 442 nm excitation, 488 nm emission		Wang [151]
rhEPO	bare fused silica	0.01 M Tricine, 0.01 M NaCl, 0.01 M CH_3COONa, 7 M urea, and 3.9 mM putrescine at pH 5.50	50μm×107(100)cm; 30 kV	low-molecular-mass excipients eliminated using Microcon-10 cartridges	UV 214 nm	H_2O, 0.1 M NaOH, H_2O, BGE	Lopez-Soto-Yarritu [137]
		80 mM morpholine, 10 mM boric acid, 8 mM NaCl, 8 mM CH_3COONa, 5.6 M urea					
	PEI coated	350 mM CH_3COOH	50μm×87(80) cm; -12 kV			0.1 M NaOH, H_2O, 5% PEI, H_2O, BGE	

Chapter 3. Review of applications in capillary electrophoresis – mass spectrometry for analysis of complex samples

3.1. Introduction

CE has been one of the analytical tools for biological sample analysis for over three decades featuring the superior separation efficiency and small solvent consumption. [152] Because the separation mechanism is complimentary for that of chromatography, which is based on mobility differences of species in electric fields, CE can provide orthogonal information obtained by chromatographic methods. In addition, the selectivity of CE can be easily varied by adjusting the BGE compositions, making it flexible and desirable for targeted analysis of complex samples.

The feasibility of coupling CE with MS was first demonstrated in 1987. [153] That adds a second dimension separation based on mass-to-charge ratio and enables faster CE analysis by reducing the dependence on the first dimensional separation. MS detectors are also more sensitive than the commonly used UV-Vis detectors. They are not as sensitive as laser induced fluorescence detectors, but they are capable of analyte identification and providing structural elucidation information.

Hyphenating CE and ESI-MS is unfortunately not as straightforward as LC-MS or GC-MS. One issue is that BGEs are restricted to the volatile ones to be compatible with ESI, resulting in some versatility loss Another concern to be addressed is the typical CE flow rate is far smaller to sustain a stable spray by conventional ESI. Complications also happen when applying a voltage at the CE inlet and another for ESI. Much effort has been devoted to the CE-ESI-MS interface developments over

the years. [154] With the commercially available or in-house made interfaces for CE-ESI-MS, analyses of protein and peptides, carbohydrates, metabolites, and other analytes have been performed.

Proteomic, glycomic or metabolomic screening or profiling, and quantification of specific analytes from complex matrices, all encounter the concentration ranges over several orders of magnitude. Their physicochemical properties are also of great diversities, which makes experiment designs difficult. Working with complex samples usually requires special sample pretreatments such as desalting or online concentration.

In this article, the applications of CE-ESI-MS to complex samples in 2007-2011 are summarized and reviewed. They are categorized into the analysis for proteins and peptides, carbohydrates, and small biomolecules according to targeted analytes. Sample preparation methods, coatings for capillary inner wall, online processing strategies, and other aspects are also reviewed in each category. It is noted that several reviews have been published in more specific areas. [155-157]

3.2. Proteins and peptides

'Proteomics' was first coined by Wilkin and coworkers in 1995. [158] It has now evolved to include studies of proteins expressed by a specific genome in cells, tissues and biofluids at any given time, including protein isoforms, co-translational modified forms and post-translational modified forms. [159] The word 'peptidomics' was introduced as the little sister or daughter omics of proteomics in 2001. [160] Similarly, it refers to the qualitative, quantitative, and functional determination of the peptidome in

a cell, tissue or organism at given time. Proteins and peptides employ similar analytical methods for elucidation given that they share mutual physicochemical properties. They can be analyzed either in their intact forms or digested by enzymes before characterization. Smaller peptides formed after enzyme digestion often behave more uniformly and less complex.LC and CE are the two main separation techniques used.

The BGE used in CE can be very close to the buffer of physiological conditions, therefore intact protein conformations can be kept as the native states during separation. Also, the inner diameter of capillary is equal or larger than 20 μm, and the commonly used ones are of 50 μm, expensive columns won't be clogged and regenerating the capillary inner surface is easy. Simply flushing the capillary between runs can eliminate the adverse effects of protein adsorption. The expense and time cost of protein/peptide analysis will therefore be lower with CE-MS. The operation time of CE-MS is usually shorter than that required in nano-LC-MS. In a recent comparison carried out by Faserl *et al.*, within only a quarter of nano-LC-MS analysis time, CE-ESI-MS identified almost 60% peptide species with comparable sample amounts applied. [161] Moreover, the superior resolving power leads to separations even for protein isoforms or post translational modifications (PTMs). The concern of CE-MS of protein/peptide analysis is that coupling CE and MS is not as straightforward as LC and MS, but in recent years different interfacing techniques have been developed, making this operation less difficult. [154]

3.2.1. Sample Pretreatment

Given the complexity of proteome and/or peptidome in cells, e.g. there are more than 20,000 proteins in the serum proteome, as well as the high salt content in biological samples, the sample pretreatment before instrumental analysis is always preferred in order to minimize the interferences and ensure reproducible instrument responses. Typical sample preparation includes liquid-liquid extraction (LLE), liquid-solid extraction (LSE) and/or solid phase extraction (SPE). In SPE, reverse phase cartridges are often used for protein/peptide analysis to get rid of the high salt content. Peptides have more predictable behaviors, while proteins are more complex. [162] Similar sample cleaning (LLE, LSE and/or SPE) techniques are also utilized after protein enzyme digestion. Various sample pretreatment methods are listed in Table 3.1.

3.2.2. Online Processing

Concentrations of proteins/peptides in biological samples span several orders of magnitude. The interested protein and/or peptide are often of lower abundance. The Achilles' heel of CE-MS has been the lower sensitivity due to smaller injection volumes, comparing to that of LC-MS. Thus, many online processing techniques have been developed to concentrate analytes as well as clean up the sample matrices. Sometimes the online processing can even be used to avoid tedious sample pretreatments. [163]

The online concentration methods for CE developed include transient isotachophoresis (t-ITP) [164], field-amplified sample stacking (FASS) [165], sweeping [166],

and dynamic pH junction [167]. Online SPE-CE-ESI-MS minimizes sample handling before the instrumental analysis. Usually an analyte concentrator (AC) is built near the inlet of capillary. The AC is microcartridge employing the same principles as SPE. A large volume of sample can be introduced to the system and eluted in a small plug with appropriate eluent. [168] Together with sample cleaning, online concentration can be achieved simultaneously. After online SPE, other processing methods can also be applied as long as proper eluent and BGE are chosen. The applications are listed in Table 3.1.

3.2.3. Capillary Coating

For protein analysis, coated capillary are commonly used because the protein-wall interaction can affect the resolution and result in peak tailing. Several types of capillary coating are used as discussed in the reviews by Haselberg *et al*. [155,169] For capillary isoelectric focusing (cIEF) coupled with ESI-MS, neutral coated capillaries are preferred, even though in some cases, focusing in bare fused silica capillaries also yielded satisfactory results, while mobilizing the cIEF effluents to MS. [28,170,171] More details are discussed in the cIEF-MS section. For peptide analysis, most applications are carried out in uncoated capillaries (Table 3.1).

3.2.4. CE-MS interface

Sheath liquid assisted CE-MS interfaces are more often utilized in protein/peptide analysis than sheathless ones. Organic solvents help to decrease the surface tension of droplets and form a stable electrospray, but to maintain the natural states of proteins requires low organic solvent portion in BGE. With adjustable sheath

liquids, researchers get more choices in BGE. Also when coated capillaries are used, sheath flow interfaces are almost exclusively employed, with only few exceptions. But the dilution effect coming along with sheath liquid has always been a problem. Some novel sheathless interfaces provide really good sensitivity for proteins and/or peptides. [172] They also show great improvement in ionization efficiency with no harm to the separation.

Table 3.1. CE-ESI-MS Analysis for Protein and Peptides in 2007-2011

Analytes	Matrix	Sample Pretreatment	Online Processing	Capillary Coating	CE-MS Interface	Mass Analyzer	Notes	Reference
Multiply phosphorylated peptides			Selective sampling	Polyacrylamide	Nano spray	Q-TOF		Ballard [173]
Angiotensin II and Leu-enkephalin			C_{18} online SPE	BFS	Sheath flow	Q		Benavente [174]
Rabit liver metallothioneins		Size exclusion filtration		BFS	Sheath flow	IT	Multiway data processing	Benavente [175]
Opioid peptides	Plasma	C_{18} SPE		BFS	Sheath flow	IT		Benavente [176]
Neuropeptides				BFS	Sheath flow	TOF		Borges-Alvarez [177]
E. Coli total proteins	*E Coli*. Lysate	Trypsin digestion	t-ITP	BFS and polyacrylamide	Sheathless	TOF		Busnel [178]
l-Phe-α-l-Asp-Gly OH				BFS	Sheath flow	Q-TOF	Peptide degradation studied at different pH	Conrad [179]
Hemoglobin acetaldehyde adducts	Blood	Enzyme Digestion		Poly-E323	Sheath flow	IT		De Benedetto [180]
Neuropeptides and tryptic BSA		ZipTip C_{18} SPE		N-methylpolyvinylpyridinuim	Sheath flow	IT	Coating first time used in CE-MS	Elhamili [181]

Analytes	Matrix	Sample Pretreatment	Online Processing	Capillary Coating	CE-MS Interface	Mass Analyzer	Notes	Reference
Zein protein fractions	Maize	LLE		EPyM/DMA	Sheath flow	IT		Erny [182]
Zein protein fractions	Flour	LSE		EPyM/DMA	Sheath flow	IT and TOF		Erny [183]
Human salivary proteins	Saliva	Dialysis, trypsin digestion and reversed-phase SPE	t-ITP	HPC	Sheath flow	Linear IT	6112 fully tryptic peptides sequenced at a 1% false discovery rate	Fang [184]
Mouse Swiss-Prot proteins	Mouse brain	Enzyme digestion, Peptide MacroTrap column SPE	t-ITP				CE then RPLC-ESI	Fang [185]
H1 histone proteins	Rat testis	LLE, endoproteinase Arg-C digestion	Stacking	M7C4I	Sheathless	Orbitrap	1/4 time for CE-MS of LC-MS	Faserl [161]
Small synthetic peptides		Labeled by 2,5-dibromo-1-ethyl-pyridinium and 2,5-dibromo-1-ethyl-thiazolium tetrafluoroborate		BFS	Nanospray	QQQ		Ferenc [186]
Aprotinin, Cyt C, chymotrypsinogen A				PEO	Sheath flow	IT		Fermas [187]

Analytes	Matrix	Sample Pretreatment	Online Processing	Capillary Coating	CE-MS Interface	Mass Analyzer	Notes	Reference
Intact ribosomal proteins	*E coli.* Lysate			BFS	Sheathless	IT and Q-TOF	BGE containing a polymer additive, allowing separation in uncoated capillaries	Garza [188]
Recombinant human erythropoietin (rHEPO) and novel erythropoiesis-stimulating protein (NESP)				UltraTol™ Pre-Coats	Sheath flow	IT		Giménez [189]
Leucine enkephalin, methionine enkephalin, dynorphin A, β-endorphin and angiotensin II	HL-1 cell lysate	C_{18} SPE	Dynamic pH junction	BFS	Sheath flow	IT	4.0×10^3–1.1×10^4-fold increase in peak intensity	Hasan [190]
Lysozyme and drug				PEI	Sheathless	TOF		Haselberg [191]
rhGH and oxytocin				PB-PVS and PB-DS-PB	Sheath flow	TOF		Haselberg [169]

Analytes	Matrix	Sample Pretreatment	Online Processing	Capillary Coating	CE-MS Interface	Mass Analyzer	Notes	Reference
Insulin, CA II, RNase A and lysozyme				PEI	Sheath flow and sheathless	TOF	Sheathless and sheath flow comparison	Haselberg [172]
α-chymotrypsinogen A, RNase A, lysozyme and Cyt C				PB-DS-PB	Sheath flow	TOF		Haselberg [192]
Neuropeptides	Plasma		C_{18} online SPE	BFS	Sheath flow	IT		Hernández [193]
Opioid peptides	Plasma		C_{18} online SPE	BFS	Sheath flow	IT	10 000-fold enhancement	Hernández [194]
α-chymotrypsin				PVA	Sheath flow	IT	Binding between protein and inhibitor studied	Hoffmann [195]
Biomarker investigation	Plasma	Tryptic digestion, ZipTip C18 SPE		MAPTAC and ODAC+TAC	Sheathless	FT-ICR		Johannesson [196]
Angiotensin I, bradykinin, neurotensin			open tubular enzyme reactor	BFS or PVA	Nanospray	TOF		Křenková [197]

Analytes	Matrix	Sample Pretreatment	Online Processing	Capillary Coating	CE-MS Interface	Mass Analyzer	Notes	Reference
Small peptides				2-acrylamido-2-methyl-1-propanesulfonic acid	Sheath flow	Q		Lu [198]
Hepcidin-25				BFS	Sheath flow	IT	CE-MS method development screening	Martin [199]
Ovocleidin-116, ovocalyxin-32, ovocalyxin-36, ovocleidin-17 and ovalbumin	Avian eggshell	CNBr/trypsin and proteinase K digestion		BFS	Sheath flow	IT	Compared with HPLC-MS	Mikšík [200]
Trypisn digested collagens		Enzyme digestion		BFS	Sheath flow	IT		Mikulíková [201]
Protein−protein and protein−metal complexes of erythrocytes	Red blood cells			PB	Sheathless	Q-TOF	The analysis of intact protein−protein and protein−metal complexes of the lysed RBC was accomplished for the first time using CE/ESI-MS	Nguyen [202]
Human AGP	Serum	Anti-AGP SPE		BFS	Sheath flow	Q-TOF	Dynamically acrylamide–pyrrolidine methacrylate copolymer (DMA-EPyM) coated	Ongay [203]

Analytes	Matrix	Sample Pretreatment	Online Processing	Capillary Coating	CE-MS Interface	Mass Analyzer	Notes	Reference
Toxic oligopeptides	Mushroom extracts	LSE		BFS	Sheath flow	IT		Rittgen [204]
Human transferrin	Serum	ProteoPrep™Blue Albumin Depletion Kit to remove albumin and IgG		PB-dextran	Sheath flow	TOF		Sanz-Nebot [205]
Protein hydrolyzates	Cosmetics	Subtilisin enzyme digestion		BFS	Sheath flow	IT		Simionato [206]
Anserine, carnosine, and buserelin	Urine	Offline preparative cITP		BFS	Sheath flow	IT	LOD after t-ITP improved by 25 times	Staňová [207]
Insulin				BFS	Sheath flow	TOF		Staub [208]
Hemoproteins	Plasma	Immunodepletion		BFS	Sheath flow	TOF		Staub [209]
Endogenous hGH, rhGH				BFS	Sheath flow	TOF		Staub [210]
rhGH, EPO				BFS and polyacrylamide	Sheath flow	TOF		Taichrib [211]

Analytes	Matrix	Sample Pretreatment	Online Processing	Capillary Coating	CE-MS Interface	Mass Analyzer	Notes	Reference
Enkephalin peptides	Cerebrospinal fluid		C_{18} online SPE	PB-poly(vinylsulfonate) bilayer	Sheath flow	IT		Tempels [212]
Microcystin	Crude algaes	LLE, C_{18} SPE	FASS	BFS	Sheath flow	Q		Tong [213]
Angiotensin II, phosphorylated angiotensin II, and insulin receptor				BFS	Nano spray	IT		Wojcik [214]
Small peptides				Polyvinylamine	Nano spray	QQQ	New coating method	Wu [215]
Peptide hormones of brain and intestine	Rat hypothalamus tissue	LLE		BFS	Sheath flow	Q		Xia [216]
peptide hormone of brain	Rat brain	LLE	t-ITP	BFS	Sheath flow	Q	40–230 fold increase in detection sensitivity	Xia [217]
Small peptides	Urine		Dynamic pH junction	BFS	Sheath flow	Q		Ye [218]

Analytes	Matrix	Sample Pretreatment	Online Processing	Capillary Coating	CE-MS Interface	Mass Analyzer	Notes	Reference
Neurotensin, neurotensin hexapeptide, cholecystokinin fragment 30–33 amide hydrochloride	Cerebrospinal fluids	C_{18} SPE	Dynamic pH junction, FASS	BFS	Sheath flow	IT	Sensitivity improved 100 fold	Ye [219]

BFS, bare fused silica; DS, dextran sulfate; EPyM/DMA, thylpyrrolidine methacrylate-N,N-dimethylacrylamide; FASS, field-amplified sample stacking; HPC,hydroxypropyl cellulose; IT, ion trap; LLE, liquid-liquid extraction; LSE, liquid-solid extraction; M7C4I, aza-1-azoniabicyclo[2,2,2]octane iodide; MAPTAC, 3-trimethylammonium propyl methacrylamide chloride; ODAC+TAC, octadecyldimethyl(3-trimethoxysilylpropyl) ammonium chloride and *N*-trimethoxysilylpropyl-*N*,*N*,*N*-trimethylammonium chloride; PB, polybrene; PEI, polyethyleneimine; PEO, polyethylene oxide; PVA, polyvinylalcohol; PVS, polyvinylsulfonate; Q, quadrupole; SPE, solid phase extraction; t-ITP, transient isotachophoresis.

3.3. cIEF-MS

In an isoelectric focusing (IEF) process, there is usually an acidic anolyte and a basic catholyte. Under an applied electric field, carrier ampholytes (CAs) will migrate to establish a pH gradient according to their isoelectric points (pIs). When amphoteric biomolecules are introduced in a mixture of CAs, they can also be focused into sharp bands around their pIs in the pH gradient. Traditional IEF is usually carried out in gel slabs. Hjerten and coworkers demonstrated that IEF can be performed in CE and proteins can be mobilized automatically to pass through the detectorin the order of pH. [220] This alternative requires less labor work, and reduces CAs and sample comsuption. The resolution is improved, while the analysis time shortened. cIEF is a promising method for the analysis of amphoteric compounds with different pIs and can be used for peptides, proteins, and protein complexes analyses. [16]

Combining cIEF and MS can lead to a more powerful biomolecule characterization platform. Both pI and molecular weight (MW) information can be provided, which is analogous to the traditional 2-D gel electrophoresis (2-DE). cIEF-MS can also offer additional qualitative and quantitative information by providing structural information by tandem mass spectrometry.

The first online cIEF-ESI-MS was demonstrated by Tang *et al.* in 1995.[221] Other feasibilities studies for improved cIEF-ESI-MS strategies have been shown in probing protein refolding [222], phosphorylation [223], glycosylation [224], screening high affinity ligands [225], monitoring intact noncovalent protein complex [226], and elucidating *E. coli*

proteomics [227-231].

cIEF is a powerful tool for biomacromolecule analysis but it has even more problems when coupled to MS. cIEF-ESI-MS processes always involve two steps: a sample and CAs mixture focusing step, and a mobilization towards MS. For each process, technical difficulties exist with interfacing cIEF to ESI-MS and various solutions have been reported to tackle these problems.

To focus samples and CAs, a pH gradient needs to be established by applying a basic and an acidic electrolyte respectively at each end of the capillary. The traditional strong inorganic acids and bases have to be replaced by volatile organic anolytes and catholytes to achieve for compatibility with ESI-MS. A more severe problem is the lack of a normal catholyte reservoir when interfacing cIEF with ESI-MS. A semi-online approach was employed in early cIEF-ESI-MS attempts. The capillary terminus was not installed to the sheath-flow ESI source but placed in a catholyte vial until the focusing was completed. [213,221,224,228] Retracting the capillary into the sheath flow tubing during focusing is another semi-online approach, and by doing that, the sheath liquid served as catholyte to provide electrical contact. [232-234] If no auxiliary gas was used in the sheath liquid interface, the capillary tip could be positioned outside the metal tubing as well. [235] Another strategy developed by Mokaddem *et al.* was to fill the terminal end of cIEF capillary with a plug of catholyte. [170,236] The required chemistry was then provided by this plug instead of sheath liquid. However, because manually switching buffer vials is required, automated operation was not easily achieved. Zhong and coworkers used an interface with a flow through

microvial [237,238] for online cIEF-ESI-MS. In their setup, both the catholyte plug and microvial catholytic reservoir methods can be used in fully automated operations (Figure 3.1).[28]

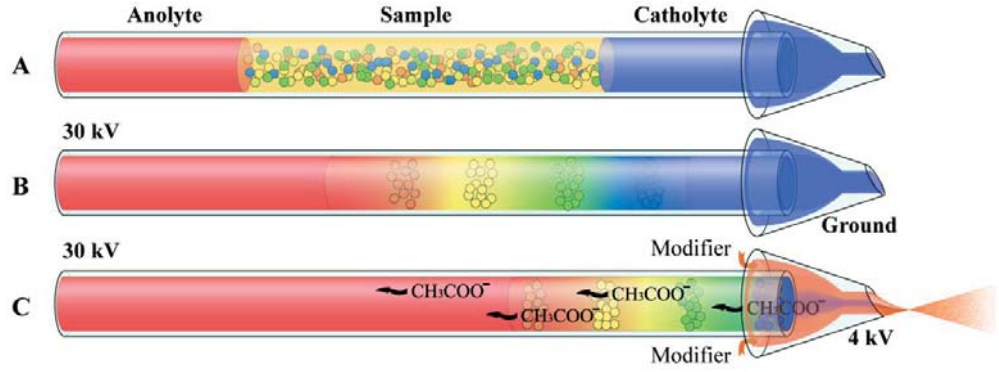

Figure 3.1. Schematic Diagram of Automated cIEF-MS
The flow-through microvial provides the electrical contact and chemical environment for cIEF. The CE-MS interface improves the ionization efficiency of the mobilized effluent without significant dilution. Reprinted with permission from "Flow-Through Microvial Facilitating Interface of Capillary Isoelectric Focusing and Electrospray Ionization Mass Spectrometry". Copyright 2011 American Chemical Society.

In the focusing step, polyethylene glycol and polyethylene oxide aqueous solutions are usually used as anti-convective medium for cIEF, which are unfortunately incompatible with ESI. Although 30% glycerol can be used to retain the high resolution in cIEF, it showed decreased solution volatility and could reduce ionization efficiency. [28,170,236,239]

Another concern needing to be addressed is that the high concentrations of CAs co-elute with analytes and compete for charges during ESI. To avoid the ionization suppression and spectral interferences, [228] microdialysis junction interfaces [240-243] or free-flow electrophoresis [240] have been reported for online CAs removal. However,

dead-volume became an issue with these methods. Mobilization of focused samples towards MS is a crucial step in cIEF-ESI-MS after focusing, unlike some on-column imaging techniques. [244] This can be achieved hydrodynamically, electroosmotically or electrophoretically. The simplest method is to use a hydrodynamic flow. The laminar flow in this situation could deteriorate resolution obtained from the focusing step. EOF mobilization is observed when bare fused silica capillaries were used, both during and after focusing. Because of the changes of pH and field strength across the capillary during and after the focusing step, it is difficult to control the EOF during cIEF. Thus, the undesirable pure EOF mobilization is usually suppressed by using neutral capillary coatings (shown in Table 3.2). Electrophoretic mobilization, also referred as chemical/anion/cation/salt mobilization, is to substitute one electrolyte after focusing to induce the mobilization under electric field. [245] No parabolic flow is introduced in this approach, and resolution achieved during the focusing step is maintained during mobilization. However, the linearity between pIs and migration times cannot be as well maintained as with hydrodynamic mobilization. All of the three mobilization methods, or any combinations of three, have been used by researchers. The mobilization methods are also listed in Table 3.2.

Table 3.2. cIEF-ESI-MS Applications

Analytes	Focusing Medium	Ampholytes	Capillary Coating	Anolyte and Catholyte	Mobilization	Reference
Myo (7.2, 6.8), CA I (pI = 6.6) and β-lac A (5.1)	water	5% pharmalytes 5-8	siloxanediopol yacrylamide double layer	1% AA as both anolyte and catholyte	Pressure moblilization	Chartogne [240]
Cyt C (9.6), Myo (7.0), CA II (5.9), Trypsin inhibitor (4.5)	water	0.5% servalytes 3-10 and isolytes 3-10 (v/v 1/1)	PVA	1% AA in 50% (v/v/) MeOH/1% NH_4OH in 50% (v/v/) MeOH	Chemical mobilzation	Clarke [233]
Cyt C (9.6),CA II (5.9),trypsin inhibitor (4.5), lysozyme (chicken egg) and Myo (7.2, 6.8),Hemo C,S,F,A	water	1% pharmalytes 3-10 and isolytes 3-10	PVA	1% AA in 50% (v/v/) MeOH/1% NH_4OH in 50% (v/v/) MeOH	Chemical mobilzation	Clarke [234]
Reduced and refold bovine pancreatic RNase A	water	0.5% pharmalytes 3-10 and 0.05% TEMED	Polyacrylamid e	20 mM PA/20 mM NaOH	Chemical combined with gravity mobilization	Jensen [222]
E Coli. Lysate	water	0.5% pharmalytes 3-10	Polyacrylamid e	20 mM PA/20 mM NaOH	Chemical combined with gravity mobilization	Jensen [230]
D.radiodurans and E.coli lysates	water	0.5% pharmalytes 3-10	Polyacrylamid e	20 mM PA/20 mM NaOH	Chemical combined with gravity mobilization	Jensen [246]

Analytes	Focusing Medium	Ampholytes	Capillary Coating	Anolyte and Catholyte	Mobilization	Reference
Angiotensin I (6.9),Angiotensin II (6.7), lysine-vasopressin (8.1), BSA (4.9), CA II(5.9), β-lac A (5.1), Myo (7.2),tetrasialo-transferrin (5.4)	water	1-2.5% pharmalytes 3-10 for angiotensin and protein standards, 1% pharmalyte5-6 and 5-8 (v/v 1/1) for CAII, tetrasialo-transferrin, β-lac)	Polyacrylamide	1% AA (pH 2.7)/0.28% NH_4OH (pH 11.2)	Chemical combined with gravity mobilization	Kuroda [235]
Myo (7.2, 6.8), CA I (6.6), β-lac (5.1)	water	1% or 2.5% pharmalytes 5-8	PVA	2% AA as both anolyte and catholyte	Pressure mobilization	Lamoree [242]
CA I (6.6,6.0), CA II (7.4)	water	1% pharmalytes 5-8	PVA	2% AA as both anolyte and catholyte	Pressure mobilization	Lamoree [243]
Myo (6.3), α-lac (4.5), β-lac A (5.25), β-lac B (5.35), BSA (4.90), a-casein (4.60), lactoferrin (8.30),Rnase A (9.45), rabbit serum	water/glycerol v/v 70:30	1% Beckman cIEF kit ampholytes 3-10 and 1% ampholine 4-6	BFS	50 mM FA and 1 mM GA (pH 2.35) in 30% (v/v) glycerol / 100 mM NH_4OH and 1 mM lysine (pH 10.6) in 30% (v/v) glycerol	Chemical mobilization combined with pressure moblilization	Lecoeur [170]
Myo (7.2), CA II (5.9), complex of src homology 2 domian and different tyrosine-phosphorylated peptides ligands	water	1% ampholine 3.5-10	PVA	0.5% AA/0.5% NH_4OH	Chemical combined with gravity mobilization	Lyubarskaya [225]

Analytes	Focusing Medium	Ampholytes	Capillary Coating	Anolyte and Catholyte	Mobilization	Reference
Creatine phosphokinase, glyceraldehyde-3-phosphate dehydrogenase,lentil lectin, hemo, β-lac A	water	1% pharmalytes 3-10	Polyacrylamide	20 mM PA/20 mM NaOH	Chemical combined with gravity mobilization	Martinovic [247]
Human alcohol dehydrogenase isoenzymes pI 8,26-8.67	water	1% pharmalytes 3-10 mixed with 1% 8-10.5	Polyacrylamide	20 mM PA/20 mM NaOH	Chemical combined with gravity mobilization	Martinovic [226]
RNase A (9.45), CA II (5.9), β-lac A (5.1), α-Tryp (8.3), Myo (6.3), trypsin inhibitor (4.5)	water/glycerol v/v 70:30	0.5-2% Beckman cIEF kit ampholytes 3-10	Bare fused silica	50 mM FA and 1 mM GA (pH 2.35) in 30% (v/v) glycerol / 100 mM NH_4OH and 1 mM lysine (pH 10.6) in 30% (v/v) glycerol	Chemical mobilization combined with pressure moblilization	Mokaddem [236]
2% red blood cell lysate	water	1% LKB 5-7	Polyacrylamide	20 mM AA/20 mM NH_4OH	Chemical combined with pressure mobilization	Severs [248]
Tryptic digests of proteins (Myo, Cyt C, bovine albumin, β-lac B, insulin, Rnase A, human albumin, CA II)	water	no ampholytes or 0.2% pharmlyte 3-10	Siloxanediopolyacrylamide double layer	1% AA as both anolyte and catholyte	Chemical mobilization combined with pressure moblilization	Storms [249]

Analytes	Focusing Medium	Ampholytes	Capillary Coating	Anolyte and Catholyte	Mobilization	Reference
Tryptic digests of *E coli*. periplasmic proteins and standard proteins (Myo, Cyt C, CA I, bovine albumin, β-lac A, Rnase A, insulin, human albumin,chicken ovalbumin, chicken lysozyme)	water	no ampholytes for E.coli periplasmic protein digests; 0.2% pharmlyte 3-10 for protein standard digests	Siloxanediopolyacrylamide double layer	0.5% AA as both anolyte and catholyte	Chemical mobilization combined with pressure moblilization	Storms [250]
Cyt C(9.6), Myo (7.2, 6.8), CAII (5.9)	water	5%-0.1% pharmalytes 3-10	Polyacrylamide	20 mM PA/20 mM NaOH	Chemical mobilization	Tang [221]
Hemo C (7.5), S (7.25), F (7.15), A (7.10)	water	0.5% pharmalytes 5-8	Polyacrylamide	20 mM PA/20 mM NaOH	Chemical combined with gravity mobilization	Tang [251]
Cyt C (9.6), RNase A (9.4), Myo (7.2, 6.8), β-lac A (5.1), CA I (6.6)	water	0.5% pharmalytes 3-10	Polyacrylamide	20 mM PA/20 mM NaOH	Chemical combined with gravity mobilization	Tang [252]
E. coli proteins	water	0.5% pharmalytes 5-8 and 3-10 (v/v 3/1)	Polyacrylamide	20 mM PA/20 mM NaOH	Chemical combined with gravity mobilization	Tang [228]
Chicken egg ovalbumin	water	0.5% pharmalytes 4-6.5	Polyacrylamide	20 mM PA/20 mM NaOH	Chemical and gravity	Wei [223]

Analytes	Focusing Medium	Ampholytes	Capillary Coating	Anolyte and Catholyte	Mobilization	Reference
Myo (7.2,6.8), β-lac (5.1), Hemo C (7.5), S (7.25), F (7.15), A (7.10)	water	0.5% pharmalytes 3-10 for protein markers and 5-8 for hemoglobin	Polyacrylamide	20 mM PA/20 mM NaOH	Chemical combined with gravity mobilization	Wei [253]
Bovine serum apotransferrin glycoforms	water	0.5% pharmalytes 5-8	Polyacrylamide	20 mM PA/20 mM NaOH	Chemical combined with gravity mobilization 8cm	Yang [224]
Hemo C (7.5), S (7.25), F (7.15), A (7.10); Cyt C (9.6), Myo (7.2, 6.8), CA II (5.9, 5.4), E.coli proteins	water	0.5 % pharmalytes 3-10 for protein standard, 5-8 for hemoglobin, 3:1 of 5-8 and 3-10 for E.coli proteins	Polyacrylamide	20 mM PA/20 mM NaOH	Chemical combined with gravity mobilization	Yang [227]
Cyt C (9.6), Myo (7.2, 6.8), CA I (6.6), CA II (5.9, 5.4)	water	0.5% pharmalytes 3-10	Polyacrylamide	10% AA/0.3% NH_4OH	Pressure mobilization	Yang [241]
E. coli lysate proteins, Cyt C (9.6), Myo (7.2, 6.8), CA II (5.9)	water	0.5% pharmalytes 3-10	BFS	0.2 M AA in 50% (v/v) MeOH /50 mM Ammonium acetate(pH 9.3)	Chemical combined with pressure mobilization	Zhang [171]

Analytes	Focusing Medium	Ampholytes	Capillary Coating	Anolyte and Catholyte	Mobilization	Reference
RNase A (9.4), Myo (7.2, 6.8), CA I (5.9), β-lac (5.1), CCK (4.1)	water/glycerol v/v 70:30	1.5% Beckman cIEF kit ampholytes 3-10	BFS and PVA	125 mM FA in 30% glycerol (v/v)/100 mM NH_4OH in30% glycerol (v/v)	Chemical mobilization	Zhong [28]

AA, acetic acid; BFS, bare fused silica; CA, carbonic anhydrase; CCK, CCK flanking peptide; Cyt C, cytochrome C; FA, formic acid; GA, glutamic acid; Hemo, hemoglobin; lac, lactoglobulin; MeOH, methanol; Myo, myoglobin; PA, phosphoric acid; PVA, polyvinylalcohol; RNase, ribonuclease.

3.4. Glycans

Glycosylation is considered as the most abundant and structurally diverse PTMs. It has tremendous consequence in causing diseases if there are genetic glycosylation disorders. [254] However, the analysis of glycoprotein and carbohydrates is still in need of drastic improvement even though protein-bound and lipid-linked oligosaccharides have been discovered for more than 50 years. [255]

Glycan analysis can be even more challenging than that of DNAs and proteins because the biosynthesis is not template-driven like DNA, RNA or even proteins. Structure variations of glycoproteins can originate from the multiple glycosylation sites of proteins as well as different glycan structures at each site. Two most abundant types of protein glycosylations are 'O-linked' to serine or threonine residues in proteins and 'N-linked' to asparagine in Asn-X-Ser/Thr sequence. Monosaccharides can link differently in enzymatic reactions to produce isomeric structures. The heterogeneity can be further increased by chemical modifications, such asphosphorylation, acetylation and sulfation. [256]

The tremendous variations in glycan structures and their biological importance require the development of new analytical methods to comprehensively characterize protein glycosylation. Several techniques have been reported in recent years, including high resolution MS, LC, CE and microarrays. [255,256] MS methods have been reported in glycan analysis but given the complexity of samples, especially the high occurrence of isomeric species, separation techniques are necessary before MS analysis is conducted. However, not many LC-MS or CE-MS methods were reported

for carbohydrate analysis until 2011. [257]

Hydrophilic interaction chromatography (HILIC) is a powerful separation technique for glycans analysis. [258] The use of silica- or amide- based stationary phases can provide good separations for different oligosaccharides. However, there are several shortcomings with HILIC-MS. One is that the analysis is rather time consuming (typically longer than 60 minutes with the column regeneration). Also for isomer discriminations, HILIC resolution is not always sufficient. The poor reproducibility is another concern, as well as the high cost of HILIC columns. Many newly developed methods have shown some improvements over the conventional HILIC-MS but they are still not completely feasible. [259] Moreover, the high effluent flow rate from LC plus the high aqueous content in the mobile phases make it difficult to maintain a stable ESI.

CE is especially suited for the separation of polar molecules with incomparable resolving power. Daves *et al.* have done a comparative study on CE and HPLC in the determination of carbohydrate-deficient transferring. The results obtained from using multicapillary electrophoresis are highly correlated with those obtained by using HPLC. [260] The separation and re-equilibration time is shorter, and the cost of capillaries is much lower than that of HILIC columns. Many people reported glycan analysis based on neutrally coated capillaries [261-265] while research employing bare fused silica capillary also yielded satisfactory results. [266]

Neutral carbohydrates sometimes cause problem in separation but many labeling methods can provide glycans with charges in solution. [261,266,267] The difficulties of

CE-MS analysis do not arise from CE, but from the ESI-MS process. Commonly negatively charged species are less ESI amenable than the positive-charged ones, so the ionization efficiency is always a concern for carbohydrate analysis. Labeling and/or indirect analytical methods can be used to alleviate the problem. Applications of using 9-aminopyrene-1,3,6-trisulfonic acid (APTS) to derivatize glycans [261,262,264,266] or with new labeling reagents 9-fluorenylmethyl chloroformate[268] and T-3 [269] have been reported. It has to be noted that, most of the labeling methods are initially developed for CE-LIF and they perform well with LIF detection. The ESI efficiency for these molecules still needs to be improved.

Similar to the protein/peptide analysis section, carbohydrate analysis in 2007-2011 is summarized according to sample preparations, capillary coatings, online processing methods, CE-MS interfaces, and applications in this period (Table 3.3). Complementary reviews are also available on glycan analysis. [156,270]

3.4.1. Sample preparation

Not too many samples directly from biological mixtures were reported in this period, and most of the applications used purified glycoprotein or glycan mixtures. The scarcity of literature shows that CE-MS for carbohydrates is still at a developing stage. Method development is of higher priority. Common steps for carbohydrates preparation is to cleave the glycans from proteins, derivatize them and then clean up by SPE.

3.4.2. Coatings for MS

Most analyses were carried out with coated capillary. Capillary inner walls were

usually modified to be near neutral and hydrophilic, often with a polyvinylalcohol coating [261,262,264,265] to eliminate the saccharide-silanol (analyte-capillary inner wall) interaction and reduce the EOF. Some uncoated capillary were also used and results are also promising. [189,266]

3.4.3. Online processing.

Since there are not too many real sample analyses in this field, special online processing techniques are not reported toward carbohydrate analysis.

3.4.4. CE-MS Interface

Almost all analyses were carried out with sheath flow interface, because it is easier to adjust the spray solution and avoid adding organic solvent in the BGE. The low flow rate aqueous phase from CE can mix with organic modifier at the CE-MS spray tips and it's easier to form stable electrospray comparing to HILIC-MS.

Table 3.3. CE-ESI-MS for Carbohydrates in 2007-2011

Analytes	Sample Pretreatment	Capillary Coating	Mass Analyzer	Notes	References
Complex human milk oligosaccharides	Graphitized carbon column SPE	BFS	IT	Sample from Breast milk and feces; CE-LIF-MS^n profiling	Albrecht [271]
Therapeutic rMAbs	PNGase F digestion, APTS labeling of released glycans, Centri-Spin 10 desalting cartridges SPE	PVA	TOF	CE−LIF−MS	Gennaro [261]
O- and *N*-glycopeptides of rhEPO	Reduction, alkylarion and enzymetic digestistion	BFS	TOF	Glycopeptide maps	Giménez [189]

Analytes	Sample Pretreatment	Capillary Coating	Mass Analyzer	Notes	References
Glycosylation of rMAbs	PNGase F digestion, APTS labeling of released glycans, Centri-Spin 10 desalting cartridges SPE	PVA	TOF		Liu [262]
APTS labeled carbohydrates		BFS	IT	CE-MS interface with a flowthrough microvial	Maxwell [266]
Fetuin, alpha1 acid glycoprotein, IgG, and transferrin	Labeled by 9-fluorenylmethyl chloroformate	BFS	IT	New labeling method, detailed *N*-glycan patterns provided	Nakano [268]
Vascular endothelial growth factor 165		Poly-LA 313	TOF		Puerta [263]
N-glycans from transferrin and fetuin	APTS labeled	PVA	Linear IT		Szabo [264]
α subunit of mouse cell line-derived recombinant human chorionic gonadotrophin (r-αhCG) and Recombinant-α hCG	Microcon Ultracel YM-3 centrifugal filter desalting	PVA	Linear IT	Relative quantitation of individual glycoforms	Thakur [265]
Glycosphingolipid analogues derived from 12-azidododecyl b-lactoside	C_{18} SPE, derivatization	BFS	IT	Sample from culture medium	Zhu [272]

APTS, 9-Aminopyrene-1,3,6-trisulfonic acid; BFS, bare fused silica; IT, ion trap; LIF, laser induced florescence; PNGase F, Peptide-N(4)-(N-acetyl-beta-D-glucosaminyl)asparagine amidase F; Poly-LA 313, polycationic amine-containing polymer; PVA, polyvinylalcohol; rMAbs, recombinant monoclonal antibodies.

3.5. Small Biomolecules

Small molecules often regulate important bioprocesses and need to be identified and quantified in complex samples. CE is used in many of these applications because most of these molecules are charged in the commonly used BGEs. Therefore, it is not surprising that research on small molecules makes up a huge percentage in applications of CE-MS, including metabolites, lipids, drugs, and food additives. [273-276] While many GC-MS [277], LC-MS [278], NMR [279], FTICR-MS [280] and enzyme based assays [281] have been carried out, CE-MS has shown to be an important complimentary method. Although it still has shortcomings such as small usable sample injection volumes, dilution effects in separation processes, CE-MS has been a methodology that can provide sufficient information about small biomolecules even in their extremely large physicochemical diversities. [157]

CE-MS has its own characteristics for analyzing biological samples, one of which is the small sample injection volumes. Usually the injected volume is in tens of nanoliters. For samples of limited amounts this is an advantage. On the other hand, the minute amount of sample injected may not give enough signals for the analysis. For method development with standard chemicals, concentration of the analytes is not an issue. However, when it comes to analyzing real samples, the range of concentrations differ significantly for different analytes depending on their own roles in the system. [282] Low concentration species could be masked in the enormous complexity of matrices. To resolve the low concentration problem, different sample preparation methods as well as on-line concentration techniques can be applied. The

sample preparation step, in addition to concentrating the targeted analyte, also eliminates interferences, making the following analysis steps (separation, ESI and MS detection) easier and more reproducible.

CE-MS has shown better promise for analyzing anionic species [283] in biological systems than other common analytical techniques. For example, the central carbon metabolism in a cell, functioning in substrate degradation, energy and cofactor regeneration, and biosynthetic precursor supply, includes glycolysis, the pentose phosphate pathway, and the tricarboxylic acid cycle. The intermediates include carboxylic acids, phosphorylated sugars, phosphocarboxylic acids, nucleotides, and cofactors, most of which are anionic. [284] The separation of these compounds is easier with the high resolving power of CE. Weaker signals obtained by MS comparing to positive species are of more concern in the CE-MS method development. To this point, it is similar to the analysis of carbohydrates. There have been several ways to solve this problem, one of which is to modify the capillary inner wall with cationic coatings [285] and separate the species under the reverse polarity to utilize the positive mode ESI, if the analytes can be analyzed in that way. Same idea with the increasing EOF is to use a pressure assisted separation. Another way is to use non-aqueous CE (NACE). Higher organic solvent content in the BGE makes it easier to achieve stable electrospray and the concern on analyte denaturing in organic solvent is not necessary here as in protein/peptide analysis. Also, derivatizing the anionic species into cationic ones can make use of positive ESI mode. [286] CE-MS interface can also be modified to fit the special need for these applications. [154]

In this section, the sample preparation techniques, capillary coatings for CE-MS, online processing methods, CE-MS interfaces, and various applications in recent five years will be summarized. Other reviews in this area can also be found in the literature. [157,276,287]

3.5.1. Sample preparation

CE-MS has been used to analyze small biomolecules for over two decades. [154] Recent reports have emphasized on sample preparation for developing more robust analytical methods. It is becoming a routine method for small molecules in real complex samples. A critical aspect for asserting biological functions of the small biomolecules is concentration. Analyte at a different concentration level may have different effect or indicate possible abnormalities of cells, tissues and/or organisms. Due to different functions or tissue matrices, concentrations of targeted molecules may vary over several orders of magnitude. To remove interferences from sample matrix and to enhance signals for target analytes, sample preparation steps are usually needed.

Common sample preparation techniques include LLE, LSE, SPE, or site specific derivatization by labeling reagents. [288,289] SPE cartridges used for small molecule analysis have more selections than those in macromolecules. Depending on the charged properties of targeted analytes, ion exchange cartridges (strong cation, strong anion, weak cation, and weak anion) can be chosen to facilitate sample preparation. For hydrophobic species, reverse phase cartridges and/or hydrophilic-lipophilic balance cartridges can also be used. The applications published during the period

reviewed are summarized in Tables 4 and 5. It is noted that processes such as protein precipitation, centrifugation, filtration, dilution, freeze dry or nitrogen dry, and reconstitution methods are not listed.

3.5.2. Coatings for MS

Bare fused silica capillaries are often used for small molecule analysis. They are simple to condition, easy to store, and inexpensive. Between runs, flushing with BGE can regenerate the inner capillary wall. Coated capillaries are used when analyte-wall interaction results in excess band broadening. If acidic BGE is used in a CE process, cationic coatings can shorten the analysis time by generating strong reversed EOF. For small molecule analysis, nearly all capillary wall coatings are ionic ones to give a strong EOF. Neutral coated capillaries are rarely used, which is quite different from protein/peptide or carbohydrate analysis. Capillary coatings include covalent bonded and dynamically adsorbed types. Over runs, covalent bonded coatings show better lifetimes while dynamic adsorbed ones require shorter preparation time.

3.5.3. Online processing

More online processing techniques are seen in the small biomolecule analysis. Similar to that in protein/peptide analysis, online SPE has been widely used. [168] FASS is also reported in some applications. [165] One nominal difference here is that pressure assisted electrokinetic injection (PAEKI) has been used in many cases. Feng *et al.* reported using PAEKI for the enrichment of nucleotide in 2006. [290] This method is also referred to as stacking or countercurrent electroconcentration.

Pressure assisted separation is also used to accelerate the analysis, because the

need for complete separation in the CE dimension is not always needed: good resolution can still be achieved by MS in the second dimension. Partial filling techniques are applied when there are nonvolatile additives in the BGE [291] for higher ionization efficiency and smaller MS background. The reported online processing methods are summarized in Tables 3.4 and 3.5.

3.5.4. CE-MS interface

Coaxial sheath liquid interfaces are the most common setup for small biomolecule CE-MS analysis. The interface allows for stable electrospray but the dilution effect by sheath liquid sometime becomes an issue for the low-concentration analytes. By using a beveled metal sprayer and a flow through micro vial configuration at the CE terminus, the stable operation region of ESI could be significantly extended. [292] Adding an atmospheric ion lens to this beveled sprayer extended the stable operational region. [293] This setup yields two to three folds increase in signal-to-noise ratio for amino acids. It could also benefit the analysis of anionic molecules with the larger acceptable range of total flow rates. Also the dilution from sheath liquid can be mitigated if the ion lens is applied.

Table 3.4. CE-ESI-MS Applications for Small Biomolecules from Human and Animals in 2007-2011

Analytes	Matrix	Sample Pretreatment	Online Processing	Mass Analyzer	Notes	Reference
Metabolomic fingerprinting	Urine			TOF		Allard [294]
β-agonists	Pork	Mixed C_8 and SCX SPE	PAEKI, NACE	TOF		Anurukvorakun [295]
Tryptophan metabolites in the kynurenic pathway				TOF	M7C4I coated capillary	Arvidsson [296]
Thiophenethylamine designer drugs	Plasma	LLE		IT		Boatto [297]
2,5-methylenedioxy derivatives of 4-thioamphetamine	Plasma	C_{18} SPE		IT		Boatto [298]
Sulfates, sulfonates and phosphates	Urine	Mixed WAX and hydrophobic cartridges SPE		Q-TOF		Bunz [299]
Ethyl glucuronide and ethyl sulfate	Serum and urine	EtG by carbon blend column SPE, EtS by WAX SPE		IT		Caslavska [300]
Amino acid and acylcarnitine metabolites	Dried blood	LSE		IT		Chalcraft [301]
Androgen glucuronides	Urine			IT	PB-DS-PB coated capillary	Cho [302]
Cocaine and metabolites	Urine			IT		da Costa [303]
Short-chain carnitines	Plasma	Dialysis		IT	10 min analysis	Desiderio [304]
Anticancer drug (imatinib)	Plasma	SCX SPE		TOF	compared LLE and SPE, SPE chosen; M7C4I coated capillary	Elhamili [305]
DNA oligonucleotides and adducts			PAEKI	Q	300-800 fold concentration	Feng [306]
Phospholipids	Rat peritoneal surface	Folch extraction	NACE	IT		Gao [307]

Analytes	Matrix	Sample Pretreatment	Online Processing	Mass Analyzer	Notes	Reference
Epinephrine, norepinephrine, dopamine, histidine and isoproterenol				TOF	Different inner diameter tested and fast separation	Grundmann [308]
Ochratoxin A		Isolated by HSA bound magnetic beads as the affinity probes		IT	Sheathless interface; APTES coated capillary	Hong [309]
Ganglioside			substitute CD with isopropyl alcohol, Na+ with NH4+	Linear IT	A liquid-junction/low-flow interface employed; PB coated capillary	Hsueh [310]
R-form LPS and lipid A	Dried bacterial cells	LLE		FT-ICR		Hübner [311]
Estrogen conjugates and regioisomers	Urine	HLB SPE		TOF		Kuehnbaum [312]
Contrast agent magnevist and transmetalation products	Plasma			TOF		Künnemeyer [313]
Alkylmethylphosphonic acids	Rat urine	LLE and SCX SPE	t-ITP	IT	40-fold sensitivity enhancement	Lagarrigue [314]
Quinolones	Chicken muscle	LSE	online SPE	IT		Lara [315]
Glutathione metabolites in oxidative stress research	Red blood cell lysate		FASS	IT		Lee [316]
Porphyrins	Urine	C_{18} SPE		IT		Li [317]
Amphetamine and related drugs	Equine plasma	LLE	NACE	IT		Li [318]
Stimulants, narcotics and their in vitro metabolites	Rat liver tissues			Q		Lu [319]
Phosphatidylethanol	Blood	LLE	NACE	IT		Nalesso [320]

Analytes	Matrix	Sample Pretreatment	Online Processing	Mass Analyzer	Notes	Reference
Metabolites in single neurons	Aplysia californica central nervous system	LLE		TOF or Q-TOF		Nemes [321]
4-alkyl 2,5 dimethoxy-amphetamine derivatives	Urine	C_{18} SPE		IT		Nieddu [322]
Antidepressants	Plasma	HLB SPE	NACE	TOF		Sasajima [323]
Ecstasy and methadone	Blood	LLE	PF-MEKC	Q		Schappler [324]
Anthocyanins	Dried calyces of Hibiscus sabdariffa L	LSE, hydrophobic SPE		TOF		Segura-Carretero [325]
Amino acids	Connective tissue of pelvic organ prolapsed patients	Reduction and acid hydrolysis	Pressure asssited separation	IT		Shama [326]
Endogenous low-hydrophilic steroids	Urine and serum	Enzyme hydrolysis, urine by Strata X/HLB SPE	PF-MEKC	QQQ		Sirén [327]
Anoic metabolites	Liver tissue	LLE		TOF	Pt spray needle improves the sensitivity; COSMO(+) coated capillary	Soga [283]
Phosphorylated species	*E coli.*	LLE	Pressure assited separation	Q	Silanol groups masked with phosphate ions by preconditioning the capillary with the background	Soga [328]

					electrolyte containing phosphate	
Analytes	Matrix	Sample Pretreatment	Online Processing	Mass Analyzer	Notes	Reference
Buserelin	Urine			Q		Staňová [329]
Isoquinoline alkaloids	Tubers of Central European *Corydalis* species	LSE	NACE	IT		Sturm [330]
Urinary nucleosides	Urine	SPE by affinity chromatography gel		Q		Wang [331]
Carboxylic acid derivatives	Rat urine	Derivatized by N-alkyl-4-aminomethyl-pyridinum iodide, WCX SPE		Q-TOF		Yang [286]
Methylene blue and its metabolites	Rat blood	LLE		Q		Yang [332]
Biodegradation products from azo dyes				IT		Zhao [333]

APTES, 3-aminopropyltriethoxysilane; CD, cyclodextrin; DS, dextran sulfate; FASS, field-amplified sample stacking; HLB, hydrophilic-lipophilic balance; HSA, human serum albumin; IT, ion trap; LLE, liquid-liquid extraction; LSE, liquid-solid extraction; M7C4I, aza-1-azoniabicyclo[2,2,2]octane iodide; MEKC, micellar electrokinetic chromatography; NACE, nonaqueous capillary electrophoresis; PAEKI, pressure assisted electrokinetic injection; PB, polybrene; PF, partial filling technique; Q, quadrupole; SCX, strong cation exchange; SPE, solid phase extraction; t-ITP, transient isotachophoresis; WAX, weak anion exchange.

Table 3.5. CE-ESI-MS Applications for Small Biomolecules in Plants and Bacteria in 2007-2011

Analytes	Matrix	Sample Pretreatment	Online Processing	Mass Analyzer	Notes	Reference
Phenolic compounds	Pollen compounds	LSE		TOF		Arráez-Román [334]
Tropane alkaloid compounds	*Atropa belladonna* L leave extract			TOF		Arráez-Román [335]

Analytes	Matrix	Sample Pretreatment	Online Processing	Mass Analyzer	Notes	Reference
Polyphenols	Almond-skin powder	LSE		TOF		Arráez-Román [336]
Acidic plant hormones	Rice leaves	LSE, C_{18} SPE and derivetized by BTA		TOF	3-aminopropyltriethoxysilane capillary	Chen [319]
Antihistamines				IT	Phosphate moving towards inlet	Chien [337]
Nicotine-related alkaloids	Gums, nicotine soft drinks, and tobacco products	LSE	NACE			Chiu [338]
Yessotoxins and pectenotoxins	Shellfish	LLE and C_{18} SPE	FASS	Q		de la Iglesia [339]
Trehalose-6-phosphate	Arabidopsis tissues	LLE, anion exchange SPE		TOF		Delatte [340]
Non-protein amino acid ornithines	Beer	Derivatized by FITC		IT		Domínguez- Vega [341]
Quinolizidine alkaloids	Dried seeds	LLE		IT		Ganzera [342]
45 metabolites	Soybean	LSE		TOF		García-Villalba [343]
Phenolic compounds	Propolis	LSE		TOF		Gómez-Romero [344]
Illicit drugs	Hair	LLE		IT		Gottardo [345]
Illicit drugs	Hair	LLE		TOF		Gottardo [346]
Melamine	Milk powder	LLE		Q-TOF		Klampfl [347]
27 metabolites	Maize	LSE		TOF	Shotgun-like approach	Levandi [348]
Phenolic acids	Virgin olive oil	diol cartridge SPE		IT		Nevado [349]

Analytes	Matrix	Sample Pretreatment	Online Processing	Mass Analyzer	Notes	Reference
Phenolic and other polar compounds	Lemon verbena extract	LLE		TOF and IT		Quirantes-Piné [350]
Chlorophenols	Honey	HLB SPE		Q		Rodríguez-Gonzalo [351]
L- and D-carnitine	Pharmaceutical products	Derivetized by 9-fluorenylmethoxycarbonyl	Succ-γ-CD	IT		Sánchez-Hernández [352]
Non-protein amino acids	Vegetable oils	LLE and derivetized by butanol		IT		Sánchez-Hernández [353]
Anionic metabolites	Physcomitrella patens	LSE	Pressure assited separation	Q	High speed and high resolution : 2 pathways	Sato [354]
Phenolic compounds	Orange peels	LSE		IT		Sawalha [355]
Amino acids	Brazil nut resin generated hydrolysates	Cation exchange resin-mediated hydrolysis		IT		Simionato [356]
Azo and methine basic dyes	Acrylic fibers	LSE		Q-TOF	Several extraction concditions compared	Stefan [357]
Tetracyclines residues	Surface water	Carbon nanotubes extration	Continuous flow system enrichment by CNT	Q		Suárez [358]
Metabolites from oxidative pentose phosphate pathway, glycolysis and Calvin cycle	Synechocystis sp. PCC 6803 and pmgA-disrupted mutant cell pellets	LLE			Polyethylene glycol coated capillary	Takahashi [359]

Analytes	Matrix	Sample Pretreatment	Online Processing	Mass Analyzer	Notes	Reference
20 metabolites	*E coli* deletion mutant	LLE		TOF	Compared with GC-MS; PolyE-323 coated capillary	Timischl [360]
Phenolic acids, procyanidins and galloylated propelargonidins	Buckwheat flour	LSE		TOF		Verardo [361]
Melamine–formaldehyde condensate				IT or Q-TOF		Vo [362]
Amino acids	Medicago truncatula liquid suspension tissue	LSE		Q	GC-MS and CE-MS compared	Williams [363]
Haloacetic acids	Tap water	Ba/Ag/H cartridge SPE	PAEKI	Linear IT	Enriched up to 20,000-fold into the capillary without compromising resolution	Zhang [364]

BTA, 3-bromoactonyltrimethylammonium bromide; CD, cyclodextrin; CNT, carbon nanotubes; FASS, field-amplified sample stacking; FITC, fluorescein isothiocyanate; HLB, hydrophilic-lipophilic balance; IT, ion trap; LLE, liquid-liquid extraction; LSE, liquid-solid extraction; NACE, nonaqueous capillary electrophoresis; PAEKI, pressure assisted electrokinetic injection; Q, quadrupole; SPE, solid phase extraction.

3.6. Conclusion Remarks

The use of CE-MS for biomolecule analysis has significantly increased in last five years. New methods are being developed for large and more complex molecules, while analyses of smaller molecules are moving toward the study in more complex tissues and matrices. Different sample pretreatment techniques, online processing methods and capillary inner wall coatings could all improve the performance of the analytical methods. Automated cIEF-ESI-MS is showing potential to replace the tedious 2-DE although more work needs to be done to achieve the robustness and reproducibility between runs. In carbohydrate analysis, CE-MS is showing great promise for being a difference-making technique in this challenging research area.

Chapter 4. Towards successful chemical mobilizations: a systematic study on the interactions among carrier ampholytes, capillary wall coatings and focusing media in isoelectric focusing

4.1. Introduction

In an isoelectric focusing (IEF) process, amphoteric molecules and carrier ampholytes (CAs) migrate to locations where the pH is equal to their isoelectric points (pIs) in a pH gradient, and are focused into sharp bands in an electric field. Traditional IEF is usually carried out in gel slabs. Hjerten and coworkers adapted this technique for capillary electrophoresis in order to minimize and automate IEF with high sensitivity. [245] Capillary isoelectric focusing avoided the labor intensive process in preparing the slab gels, reduced CAs and sample consumptions, provided improved resolution and shortened the analysis time.

Two types of detection methods can be used in capillary isoelectric focusing (cIEF): whole column imaging [244] or single-point detection. In the single-point detections, resolved sample bands are mobilized to the detector sequentially. It can be achieved by concurrent focusing with the EOF mobilization, i.e., one-step cIEF [27], or in two steps: focusing and subsequent mobilization. The two-step mobilization can either be achieved hydrodynamically via applying pressure/vaccum/gravity to one end of the capillary [24-26] or electrophoretically[18,28,29,365] (also called chemical/cathodic/anodic/salt mobilization) by replacing the catholyte or anolyte with a chemical mobilizer to induce the mobilization. Hydrodynamic mobilization is

applicable for almost all cIEF experiments. It maintains the linearity of pH gradient formed in the focusing, but the resolution will be reduced by the presence of laminar flow. [366] The electrophoretic mobilization maintains the resolution albeit at the expense of reduced pH gradient linearity. In many cIEF experiments employing hydrodynamic mobilization, voltages are also applied to mitigate the effect of the parabolic flow. [26,170,228]

Electrophoretic mobilization is not always the first choice, despite its higher resolution: it may lack one or more of the aforementioned advantages of hydrodynamic mobilizations. Furthermore, unpredictable difficulties may arise, such as dynamic interactions between CAs and capillary inner walls. Tang and coworkers found CAs could affect EOF mobilities. [367] Zhang *et al* .[171] studied the dynamic coatings, where an anodic EOF was induced between Pharmalytes and bare fused silica (BF) capillary surfaces. In this case, the catholyte would be pumped into the capillary by the anodic EOF, resulting in no peaks detected under normal polarity. The current profile was also similar to those used in successful chemical mobilizations. However, with the same type of CAs and capillary, electrophoretic mobilization was achieved by adding a small amount of methylcellulose. [228] This shows that CAs behave differently in different media and/or coated capillaries.

Capillary wall coatings and additives in sample mixtures are also important for cIEF. Adsorptions of biomolecules to the capillary inner walls adversely affect the resolution and reproducibility of cIEF, so coated capillaries are usually used, [269,368] although sometimes uncoated fused silica capillaries are also applied[18,24,28,171,228,365].

Capillary coatings, either hydrophobic or hydrophilic, used in cIEF experiments include but are not limited to: polyacrylamide [369], polyvinylalcohol (PVA) [370], hydroxypropylcellulose (HPC) [25], dimethylpolysiloxane (DB-1) [88], fluorocarbon (FC) [371] and other polymer materials [372]. They are either covalently bonded or dynamically adsorbed to the silanol groups. Additives like methylcellulose [373] and hydroxypropylmethylcellulose [374] are added to reduce the analyte-wall interactions. The capillary coating and/or additives also suppress the EOF which is conventionally believed to decrease the focusing resolution. Other than water medium, experiments were also carried out in some anti-convective media such as polyethylene glycol [29,375] and glycerol [28,170].

In this work, a systemic study on the interactions among CAs, sample media and capillary coatings was carried out to provide guidance for choosing optimal combinations for successful focusing and electrophoretic mobilizations. Combinations of four designer broad range CAs (Fluka 3/10, Servalyt 3/10, Pharmalytes 3/10 and Bio-lyte 3/10), and six types of capillaries (BF, PVA, HPC, DB-1, FC and N-CHO), were evaluated in terms of EOF mobilities. Tests were conducted under different CA concentrations in three sample media (water, 30% glycerol and 50% Beckman cIEF polymer gel (cIEF gel)). At the 0.1%-1% (w/v) CA concentration range, a small forward EOF ensures a higher chance of good focusing and successful electrophoretic mobilization, while a reverse EOF will deteriorate the separation and hinder mobilization. Applicable combinations of capillary coatings, types of ampholytes and media are summarized. Comparison between cIEF separations conducted with HPC

and BF capillaries, with 0.64% (w/v) Bio-lyte, demonstrated the effects of neutral and negative (reverse) EOF, respectively.

4.2. Experimental Section

4.2.1. Chemicals and Materials

All chemicals were of analytical grade or better and used without further purification. Caffeine, glycerol (>= 99%, for electrophoresis), iminodiacetic acid (IDA), L-arginine (98%, Arg), Pharmalytes (pH 3-10) and PVA were obtained from Sigma-Aldrich (St. Louis, MO). Sodium hydroxide (NaOH), formic acid (FA) and acetic acid (AA) were bought from Fisher Scientific (ON, Canada). HPC (MW 100,000) was purchased from Scientific Polymer Products (Ontario, NY). Fluka ampholytes (pH 3-10), synthetic peptide pI markers (pI 9.5, 7.0, 5.5 and 4.1) and cIEF Gel Polymer solution were provided by Beckman Coulter (Brea, CA). Bio-lyte (pH 3-10) and Servalyt (pH 3-10) were obtained from Bio-Rad Laboratories (ON, Canada) and SERVA Electrophoresis (Heidelberg, Germany), respectively. All the solutions prepared were filtered through 0.22 μm membranes (Millipore Co., Billerica, MA) before use.

The length, inner diameter and outer diameter of all the capillaries used in EOF evaluation and cIEF analysis were 40 cm (30 cm effective length), 50 μm and 360 μm, respectively. BF capillaries were purchased from Polymicro Technologies (Phoenix, AZ). DB-1 and FC (μSIL-FC) capillaries were bought from Agilent Technologies (Santa Clara, CA). N-CHO capillaries were obtained from Beckman Coulter. PVA and

HPC coated capillaries were prepared according to the procedures reported in literatures. [25,376]

All experiments were performed with a Beckman Coulter PA 800 plus capillary electrophoresis system (Brea, CA). Time programs were carried out by 32 Karat software (Beckman Coulter). Sigma Plot 9.0 (Systat Software, San Jose, CA) was used for data processing and figure plotting.

4.2.2. EOF Evaluations

All capillaries were flushed by water and conditioned overnight before use and stored in water. EOF mobilities of BF, DB-1, FC, PVA, HPC, N-CHO capillaries were measured according to the method described in literature [377]. 0.1 M caffeine was used as the neutral marker. CA stock solutions were diluted to the concentration series of 0.0001%, 0.001%, 0.01%, 0.1% and 1% (w/v) by water, glycerol/water 3:7 (v/v) and cIEF gel/water 1:1 (v/v). The capillary and vials at two ends were filled with diluted ampholyte solutions prior to each run. The EOF evaluation was repeated three times for each condition with specific medium, capillary coating, ampholyte brand and concentration. The detection wavelength was 200 nm. Temperature was controlled at 25 ºC. Averages of three runs were plotted as functions of CA concentrations.

4.2.3. cIEF

BF and HPC capillaries were selected to perform the cIEF experiments. The anolyte, catholyte and chemical mobilizer were 0.1 M FA, 0.3 M NaOH and 0.35 M AA, respectively. The peptide pI marker mixture provided by AB SCIEX Separations

(Brea, CA, USA) was prepared in 30% glycerol with 0.64% (w/v) Bio-lyte, 1.2 mM IDA and 23 mM Arg. All four pI markers were used in BF capillary cIEF run and 3 of them (pI 7, 5.5, 4.1) for the HPC capillary. The capillaries were first flushed by water for 5 min at 40 psi , and then fully filled with sample mixture by applying 25 psi for 60 s from the inlet. Focusing was carried out by applying 30 kV across the capillary with the anode in the anolyte and the cathode in catholyte for 15 min. After the current decreased to minimum, the catholyte was replaced by the chemical mobilizer and 30 kV potential was continued for another 60 min.

Experiments with counter-pressures against reversed EOF at 0, 0.1 and 0.2 psi during the focusing step were performed with the BF capillary. After the focusing completed, instead of applying voltage, 1 psi forward pressure was used to push the peptide train through the detection window. The detection wavelength was 280 nm and analysis temperature was 25 ºC for all cIEF experiments.

4.3. Results and Discussions

EOF, forward or reverse, is usually believed to decrease the resolution during the focusing step. Various attempts were made to reduce EOF: using coated capillaries and/or applying polymer additives to sample solutions. A negative EOF was thought to be the cause for the poor focusing of proteins as well as hindering the cathodic electrophoretic mobilization [171], which is also demonstrated later in this chapter. However, a positive EOF with a reasonable magnitude [372] yields adequate resolutions and facilitates the mobilization of focused zones [27,28].

cIEF experiments were performed with different designer CAs and various types

of capillaries, and only some of them achieved successful electrophoretic mobilizations. Since the CAs and additives are able to form dynamic coatings with the BF capillaries [171], EOF would be varied due to the interaction [367]. Some dynamic interactions probably do happen between CAs, sample media and the capillary coatings. Therefore, with the four common brands of broad range CAs, and widely used types of capillaries, EOF mobility evaluations were carried out. Commonly used media for cIEF (water, glycerol and cIEF gel) were tested.

4.3.1. EOF evaluations using water as medium

Figure 4.1 shows the EOF mobilities at different concentrations of Fluka (1A), Servalyt (1B), Pharmalytes (1C) and Bio-lyte (1D) in water without additives. The BF, PVA, HPC, DB-1, NCHO, and FC capillaries were evaluated. When the concentration of CAs are below 0.1%, EOF can be drastically different with the type of CA used. This phenomenon could be explained by two reasons. First, the dynamic interactions between the CAs and the capillary inner wall influence the magnitude of EOF mobility. [367] Second, ionic strength changes due to dilution also affect EOF. Since the CA solution is also a buffer system [378], greater dilution leads to stronger dissociation; the ionic strength of the BGE will not decrease in the same magnitude as the concentration does. The two factors interact with each other and yield the fluctuating EOF trends at 0.0001%-0.1% (w/v) range. Both quantification of the dissociation equilibrium and elucidation of CAs-inner wall interaction mechanism are difficult to achieve without sufficient information. Unfortunately, all the compositions and structures of CAs are proprietary. However, at the higher concentrations, which is the

common working CA concentration range for cIEF experiments, EOF changes corresponding to the CA concentration changes seem not as drastic.

In Figure 4.1, when the medium is water, most combinations of CAs and capillaries have positive EOFs at the common working range (0.1%-1%, w/v). At each concentration, tests were repeated three times. The averages of three runs were plotted as functions of ampholyte concentrations. Only when applying Pharmalytes or Bio-lyte with the BF capillary, negative EOFs are observed, which means chemical mobilization is not possible with those combinations. The positive EOFs are too strong to produce a good focusing either, because at larger than 2×10^{-4} $cm^2V^{-1}s^{-1}$ [372] when using Servalyt, Pharmalytes or Bio-lyte with the FC capillary, focused peaks can be broadened. Feasible combinations for chemical mobilizations are listed in Table 4.1.

4.3.2. EOF evaluations using glycerol/water 3:7 (v/v) as medium

30% glycerol can eliminate the EOF to the same extent as other supporting media, such as cIEF gel. [239] Compared with other polymer additives, glycerol is of smaller molecular weight (MW) and lower boiling point. Glycerol also stabilizes protein structures. These properties make 30% glycerol a good substitute for gels made from polymers in cIEF-ESI -MS experiments. [28,170] cIEF-ESI-MS is a promising alternative to the traditional 2-D gel electrophoresis which characterizes the biomolecules by their pIs and molecular weights simultaneously.

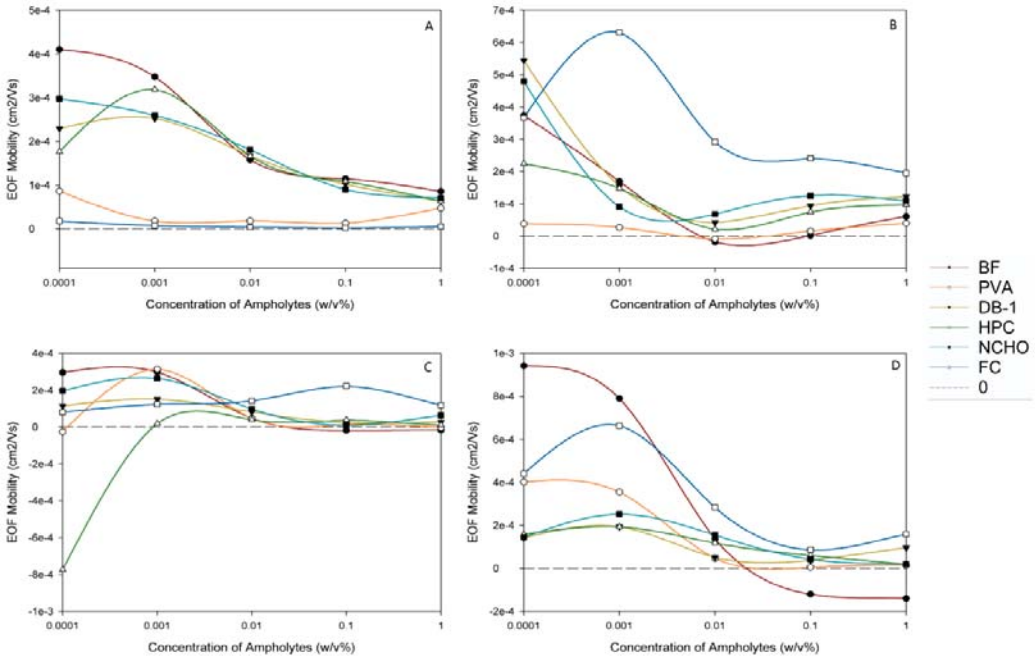

Figure 4.1 EOF measurements at different concentrations of carrier ampolytes in water.

Within the figure, Panels A: Fluka, B: Servalyt, C: Pharmalytes, and D: Bio-lyte. Line notations: BF, bare fused silica capillary; PVA, polyvinylalcohol coated capillary; HPC, hydroxypropylcellulose coated capillary; DB-1, dimethylpolysiloxane capillary; FC: fluorocarbon capillary.EOF was tested according to literature reported method[377] in the concentration series of 0.0001%, 0.001%, 0.01%, 0.1% and 1% (w/v) for all ampholytes. Error bars are not showing for the clarity of the figure.

In the 30% glycerol medium, again the 0.1%-1% (w/v) range is more important. As shown in Figure 4.2, 30% glycerol suppresses EOFs very well. All the EOF mobilities are smaller than 2×10^{-4} $cm^2V^{-1}s^{-1}$ and most of them are at least 10 times smaller than those in water. Fluka (Figure 4.2A) performs better over others regarding the EOF positivity and magnitude. All the combinations displayed flat trends at high CA concentrations. Only the FC capillary is incompatible with Fluka according to the forward-EOF standard. Servalyt (Figure 4.2B) is not an ideal choice for FC capillary

either, for the same reason. But with other types of capillaries, Servalyt (Figure 4.2B) can be a good choice for cIEF experiments with electrophoretic mobilization, given all EOFs are to the positive direction. The shortcoming of Servalyt compared with Fluka is that the magnitude of EOFs are larger (still within acceptable range) and the changes corresponding to varied concentration are more dramatic with Servalyt at concentration range 0.1%-1%.

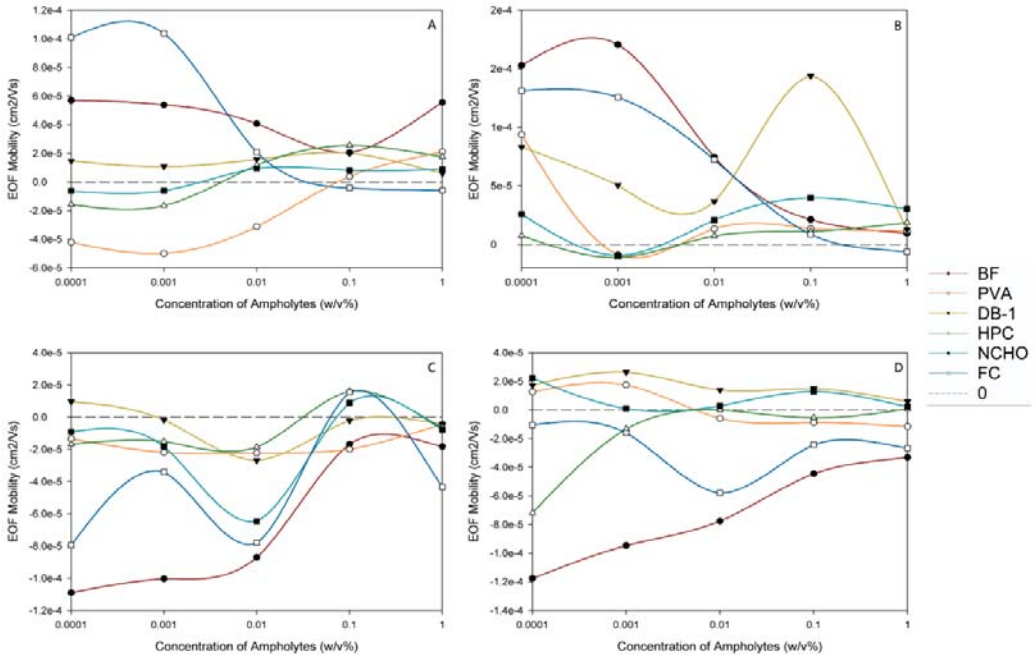

Figure 4.2. EOF measurements at different concentrations of carrier ampolytes in 30% glycerol

Panels A: Fluka, B: Servalyt, C: Pharmalytes, and D: Bio-lyte. Line notations BF: bare fused silica capillary; PVA: polyvinylalcohol coated capillary; HPC: hydroxypropylcellulose coated capillary; DB-1: dimethylpolysiloxane capillary; FC: fluorocarbon capillary.
EOF was tested according to literature reported method[377] in the concentration series of 0.0001%, 0.001%, 0.01%, 0.1% and 1% (w/v) for all ampholytes. At each concentration, tests were repeated three times. Averages of three runs were plotted as functions of ampholyte concentrations. Error bars are not showing for the clarity of the figure.

For Bio-lyte (Figure 4.2D), when used in the 30% glycerol medium, successful electrophoretic mobilization may happen with DB-1 or N-CHO capillaries. It is also possible with the HPC capillary because in the EOF measurements at 0.1% and 1% (w/v) concentration, the direction of EOF swings between forward and reverse (results not shown). And the mobility is quite small (at 10^{-6} $cm^2V^{-1}s^{-1}$ order level) so EOF with Bio-lyte – HPC combination can be treated as neutral in this case. And this possible electrophoretic mobilization is also confirmed in later discussion. For Pharmalytes (Figure 4.2C), the EOF measurement didn't show promise for the application with 30% glycerol: some of EOF mobilities are negative while for some others the direction changes between forward and reverse in the concentration range of 0.1%-1%, making it hard to predict whether the experimental conditions will yield satisfactory results or not.

The EOF measurements were carried out in one specific capillary and with one particular batch of CAs at a time. There might be small variations in capillary inner wall properties and/or CA composition if different batches are tested. That's also why the smoother changing trend is emphasized in the text – only when it's not changing dramatically, slight variations of concentrations will not bring great changes in EOF mobilities. Feasible combinations of CAs and capillary coatings are summarized in Table 4.1.

Another interesting result from using a 30% glycerol medium is that not only the magnitudes of EOF mobilities decrease, but different trends from those in water medium, corresponding to the concentration changes, are also observed (Figure 4.1

and 4.2). This could be caused by the modification of inner capillary walls by glycerol or the interaction between glycerol and CAs. It is also possible that both processes happen simultaneously. With the insufficient information on the composition of CAs and capillary coatings, the interaction mechanism cannot be fully understood.

4.3.3. EOF evaluations using cIEF gel/water 1:1 (v/v) as medium

Various concentrations of cIEF gel have been used in cIEF experiments as medium or additives. [29] The EOF evaluation results using cIEF gel are shown in Figure 3. Similar to 30% glycerol, the 50% gel eliminates EOF mobilities are smaller than 2×10^{-4} $cm^2V^{-1}s^{-1}$. [372] Fluka (Figure 4.3A) still exhibits compatibility with different capillaries. All combinations between Fluka and capillaries display forward EOF in cIEF gel, in working concentration range. For Bio-lyte (Figure 4.3D), only N-CHO capillary is appropriate for the successful electrophoretic mobilization. Other coatings either yield negative EOFs in 0.1-1% (w/v) range or have their turning points between positive and negative EOFs in that region. Similar scenarios occur with Servalyt (Figure 4.3B), where the most suitable capillary is HPC. In our study, Pharmalytes (Figure 4.3C) fail to show possibility for electrophoretic mobilization if the medium was 50% cIEF gel.

Again, different trends from those in water are observed in gel medium (Figure 4.1 and 4.3), indicating there is some CA-medium-capillary coating interaction. The components of cIEF gel include ethylene glycol (EG) and polyethylene oxide (PEO). [29,375] Even though similar interaction properties should be predicted from homologous EG and glycerol, the trends in 30% glycerol and 50% cIEF gel are not the same

(Figure 4.2 and 4.3). This means that other components, e.g. PEO, also play essential roles in the dynamic interaction. [375] Unfortunately the interaction cannot be fully elucidated without more information of those products.

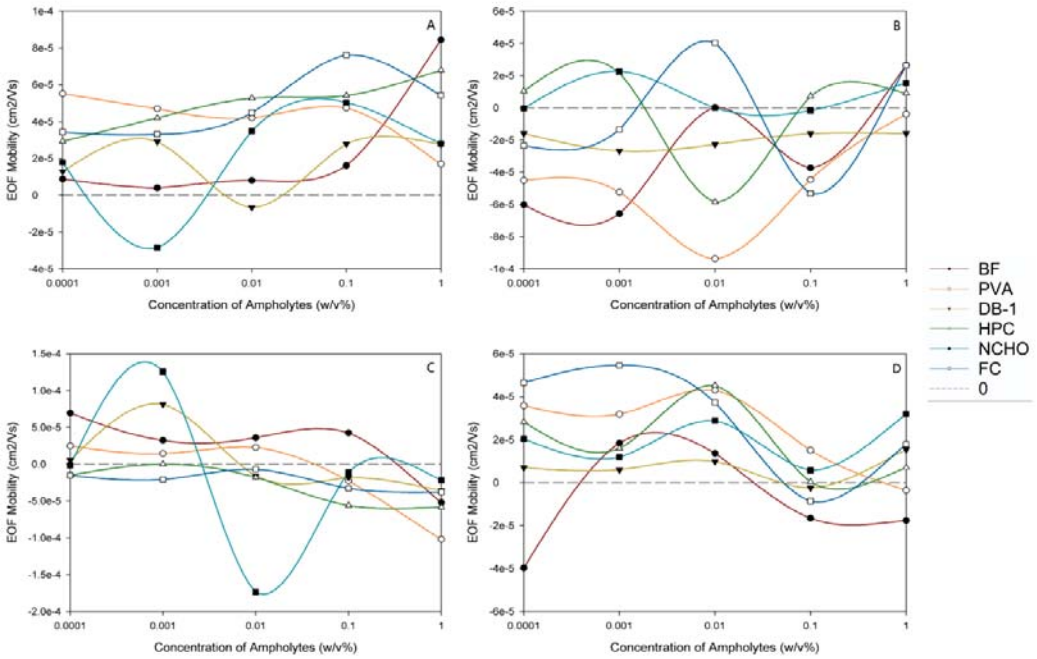

Figure 4.3. EOF measurements at different concentrations of carrier ampolytes in 50% Beckman cIEF polymer gel.

Panels A: Fluka, B: Servalyt, C: Pharmalytes, and D: Bio-lyte. Line notations BF: bare fused silica capillary; PVA: polyvinylalcohol coated capillary; HPC: hydroxypropylcellulose coated capillary; DB-1: dimethylpolysiloxane capillary; FC: fluorocarbon capillary.
EOF was tested according to literature reported method27 in the concentration series of 0.0001%, 0.001%, 0.01%, 0.1% and 1% (w/v) for all ampholytes. At each concentration, tests were repeated three times. Averages of three runs were plotted as functions of ampholyte concentrations. Error bars are not showing for the clarity of the figure.

4.3.4. Choices for carrier ampholytes, medium and capillary coating combinations

Based on EOF evaluation results, choices of CA, medium and capillary coating

combinations are summarized in Table 4.1. The averages of magnitudes of EOF at 0.1% and 1% (w/v) were calculated and evaluated. The combinations which yield a weak enough EOF will help to achieve good resolution for focusing. The average at the range from -1×10^{-6} $cm^2V^{-1}s^{-1}$ to 1×10^{-6} $cm^2V^{-1}s^{-1}$ is treated as neutral and labeled as '0' in Table 4.1. Those neutral combinations will supply good resolutions as well as electrophoretic mobilization possibilities. The positive ones within 1×10^{-6} $cm^2V^{-1}s^{-1}$ to 2×10^{-4} $cm^2V^{-1}s^{-1}$ range are believed to be good candidates for successful electrophoretic mobilizations after focusing, and labeled as '+' in the table. All other combinations are labeled as '-' in Table 4.1 either because of the strong negative EOF or the existing turning point between forward and reverse EOFs in 0.1%-1% (w/v) range. Also, the combinations demonstrating positive EOF mobilities over 2×10^{-4} $cm^2V^{-1}s^{-1}$ are in this category. These combinations are not recommended for cIEF experiments with electrophoretic mobilizations.

4.3.5. cIEF experiments by electrophoretic mobilization under neutral and negative EOF conditions

To prove our hypothesis that when a reverse EOF forms from the CA-medium-capillary interaction, there will be neither a high chance for effective electrophoretic mobilization nor good focusing, cIEF experiments for Beckman synthetic peptide pI markers were carried out with 0.64% (w/v) Bio-lyte in 30% glycerol with the BF capillary. That combination displays a negative EOF according to our EOF evaluations.

Table 4.1. Summarization of Possible Choices of Carrier Ampholyte-Medium-Capillary Coating Combinations for cIEF with Chemical Mobilization

	Water				30% Glycerol				50% cIEF Gel			
	A	S	B	P	A	S	B	P	A	S	B	P
BF	+	+	-	-	+	+	-	-	+	-	-	-
PVA	+	+	+	+	+	+	-	-	+	-	-	-
HPC	+	+	+	+	+	+	0	0	+	+	0	-
DB-1	+	+	+	+	+	+	+	0	+	-	+	-
N-CHO	+	+	+	+	+	+	+	-	+	+	+	-
FC	+	-	-	-	-	-	-	-	+	-	-	-

Abbreviations: A: Fluka; S: Servalyt; B: Bio-lyte; P: Pharmalytes. BF: bare fused silica; PVA: polyvinylalcohol; HPC: hydroxypropylcellulose; DB-1: dimethylpolysiloxane; FC: fluorocarbon.
The averages of EOF mobilities at 0.1% and 1% (w/v) were calculated and evaluated. The combinations which yield a weak enough EOF will help to achieve good resolution for focusing. The positive ones within 1×10^{-6} $cm^2V^{-1}s^{-1}$ to 2×10^{-4} $cm^2V^{-1}s^{-1}$ range are labeled as '+'. The average at the range from -1×10^{-6} $cm^2V^{-1}s^{-1}$ to 1×10^{-6} $cm^2V^{-1}s^{-1}$ is labeled as '0'. All other combinations are labeled as '-', either because of the strong negative EOF or due to the existing turning point between forward and reverse EOFs in the 0.1%-1% (w/v) range. Also, the ones with positive EOF mobilities over 2×10^{-4} $cm^2V^{-1}s^{-1}$ are labeled as '+' or '0' yield higher chances for successful chemical mobilization.

For comparison, the same experiment was repeated with a HPC capillary which has a near neutral EOF, as measured. Figure 4.4 shows the electropherogram at 280 nm with the HPC capillary. All three pI markers (pI 7.0, 5.5 and 4.1) are focused nicely, suggested by the sharp peaks. In addition, the mobilization is accomplished

fully by voltage after replacing the catholyte with the chemical mobilizer. However, with the BF capillary, due to the reverse EOF, no peaks appeared after 70 mins of chemical mobilization. None went through the detection window even after applying pressure assisted (1 psi) chemical mobilization for another 20 mins after having pure chemical mobilization for 60 mins. If both voltage (30 kV) and pressure (1 psi) were applied at the beginning of mobilization, only a fluctuating baseline was observed (results not shown). Those results suggest the chemical mobilization is not possible under negative EOFs. Therefore, for cIEF with BF capillary, results displayed in Figure 4.5 were obtained by pure pressure mobilization.

In Figure 4.5, three electropherograms are shown for BF capillary cIEF of four pI markers. During focusing step, no pressure, 0.1 psi and 0.2 psi forward pressure were applied to counterbalance the reverse EOF. When there was no counter pressure applied during focusing, the negative EOF deteriorated the process: 5 peaks appeared because of the insufficient merging of cathodic and anodic peaks. [379,380] Employing 0.1 psi counter-pressure focused the bands better, although peaks were not as sharp as the ones obtained with HPC. When the counter pressure was 0.2 psi, which was too high for the process, the resolution was adversely affected again. This could be interpreted as the presence of laminar flow as well as large forward flow rate, which is just as bad as the negative EOF. The series of different counter-pressure-assisted cIEF experiments verify that under strong negative EOF conditions, focusing of sample bands cannot be achieved.

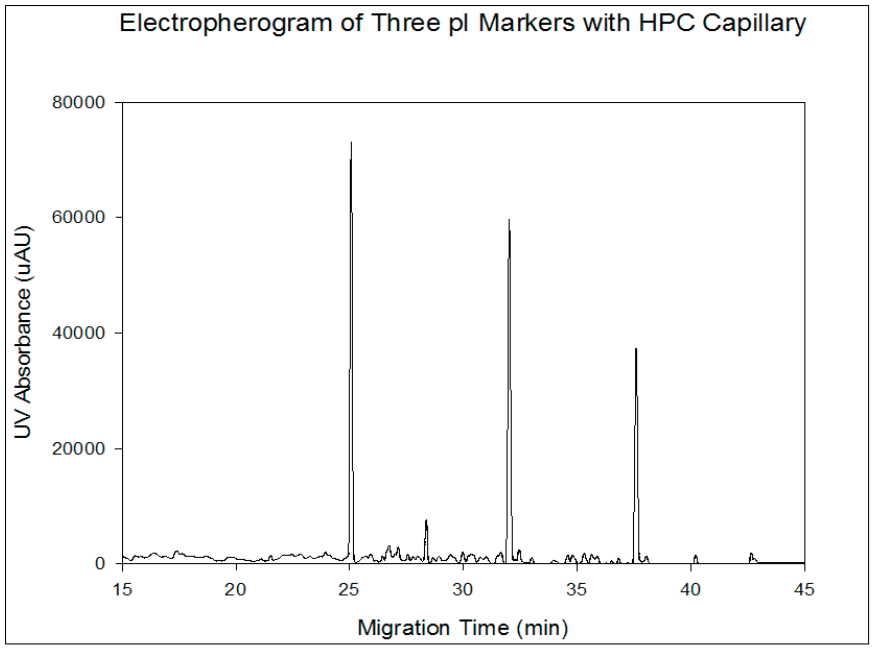

Figure 4.4. Electropherogram of Three pI Markers by Chemical Mobilization with HPC

Some successful focusing and/or chemical mobilizations were reported in literatures under negative EOFs. [18,365,381] But none of them were without additives like methylcellulose or hydroxypropylmethylcellulose. This is because adding those polymers has already modified dynamic interaction discussed in our previous EOF measurements. These polymers are believed to form a dynamic coating at the capillary inner walls [25] which afterwards possess similar characteristics as HPC capillary, where the EOF is positive. Therefore those successful results are supporting our hypothesis in another way. Moreover, the polymer additives, due to the high viscosities, would also reduce the magnitude of the negative EOF. With these additives, the reverse EOF became near-neutral, which could be another explanation

for the successful chemical mobilizations.

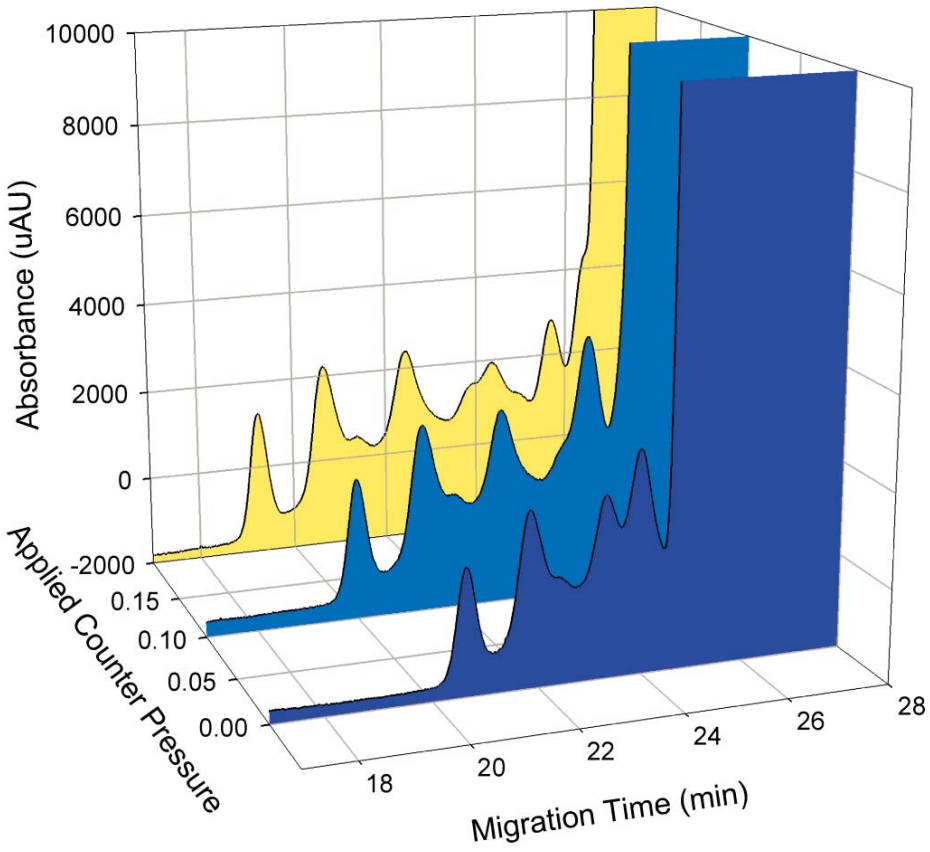

Figure 4.5. Waterfall electropherograms of four pI markers by pressure mobilization with BF capillary under different counter pressures during focusing

4.4. Concluding remarks

Carrier ampholytes and focusing media can form a dynamic coating with the capillary inner wall. Glycerol and Beckman cIEF gel play important roles in modifying capillary inner walls. The concept of ampholyte-inner wall interaction is extended to ampholyte-medium-inner wall interaction in order to account for the influence of medium on EOF, which we have demonstrated here. Only when the EOF

magnitude is within a certain range could acceptable focusing and electrophoretic mobilization be achieved. Based on the EOF measurement results, suggested conditions for choosing CAs, media and/or capillary coatings are summarized.

Chapter 5. Potential of Capillary Isoelectric Focusing Mass Spectrometry for Precise Determination of Isoelectric Point

5.1. Introduction

Capillary isoelectric focusing (cIEF) is an important analytical tool for the separation and characterization of biomolecules. Minute structural changes on a large protein, such as phosphorylation, glycosylation and other types of post-translational modifications (PTM), can result in changes in isoelectric point (pI), and thus the various isoforms can be separated by cIEF.[16] Coupling cIEF with mass spectrometry (MS) can add a second-dimension separation based on mass-to-charge ratios (m/z). cIEF-MS provides analogous information (i.e. pI and molecular weight) to that obtained from two dimensional gel electrophoresis (2-DE) but requires a smaller sample and less labor.[269] Several papers have shown the feasibility of directly coupling cIEF to MS. [230,251,382,383] We have reported previously an online cIEF-ESI-MS method facilitated by a flow-through micro vial CE-MS interface.[28] The work demonstrated that it is possible to mobilize the pI markers chemically without applying pressure to push the focused protein train through, avoiding decreased resolution due to laminar flow. Furthermore, the fully automatic operation does not require any manual switches, making online cIEF-MS a more promising alternative to the traditional, laborious 2-DE. With the use of tandem MS for the identification of the sites and the nature of the PTM [384], it could be feasible to identify

minor changes of protein therapeutics during large scale production with the highly laborious purification processes current required in the biopharmaceutical industry.

A pH gradient can be established by the migration of carrier ampholytes (CAs) in an electric field and CA-formed Gaussian peaks overlap with each other. To understand the cIEF process, computer simulations were carried out previously.[385] However, no experimental data has been obtained to prove how effectively the CAs are focused, how they align along the column, or how well the pH gradient is maintained during the mobilization step. In this work, using the flow-through micro vial CE-MS interface[237,292,293,386], the shape of commercially available CA bands is directly observed by MS. cIEF has been used to determine the pIs of biomolecules and/or particles.[387-392] Shimura *et al.* introduced low mass peptide markers for more precise pI determination and since the detection time vs. pH relationships were not always linear, linearity was only assumed between 2 adjacent marker peptides.[390] In addition, those peptides are not good pI marker candidates for cIEF-MS due to the spectral interferences at m/z below 1000. Applying a similar concept, the directly observed designer CAs in cIEF-MS could enable more precise biomolecule pI determinations by using the CA peaks as more finely spaced internal pI markers.

5.2. Experimental section

5.2.1. Chemicals and Materials.

All chemicals were of analytical grade or better and used without further purification. Fluka ampholytes (pH 3-10), glycerol (>= 99%, for electrophoresis),

immunoglobulin G from rabbit serum (IgG), methanol and Pharmalytes (pH 3-10) were obtained from Sigma-Aldrich (St. Louis, MO). Ammonia hydroxide, formic acid (FA) and acetic acid (AA) were purchased from Fisher Scientific (ON, Canada). Hydroxypropyl cellulose (HPC, MW 100,000) was purchased from Scientific Polymer Products (Ontario, NY). Bio-lyte (pH 3-10) was purchased from Bio-Rad Laboratories (ON, Canada). Myoglobin and Servalyt (pH 3-10) were obtained from SERVA Electrophoresis (Heidelberg, Germany). β-lactoglobulin (β-lac), CCK flanking peptide (CCK) and Ribonuclease A (RNaseA) were obtained from Beckman Coulter (Brea, CA). All capillaries were purchased from Polymicro Technologies (Phoenix, AZ).

The anolyte, catholyte and mobilizer used in all experiments were 0.1 M FA (*aq*), 0.1 M NH_4OH (*aq*) and 0.1 M AA in 50% methanol (v/v), respectively. The sample mixtures were all in 30% glycerol (v/v). For the pI marker analysis, the sample was a mixture of β-lac (0.05 mg/mL), CCK (0.025 mg/mL), myoglobin (0.125 mg/mL), RNaseA (0.3 mg/mL) and 0.4% Fluka (w/v). For Fluka, Bio-lyte and Servalyt characterizations, the samples were mixtures of 0.4% respective CAs (w/v), 0.025 mg/mL CCK and 0.3 mg/mL RNaseA. For Pharmalytes characterization, the ampholytes concentration was 1.6% (w/v). The final concentrations in the unknown protein pI test sample were 0.025 mg/mL of CCK, 0.3 mg/mL of RNaseA, 0.4 mg/mL IgG and 0.4% Fluka (w/v). All the solutions prepared were filtered through 0.22 μm membranes (Millipore Co., Billerica, MA) before use.

5.2.2. Capillary Isoelectric Focusing-Mass Spectrometry

cIEF was performed on a MDQ Capillary Electrophoresis System (Beckman Coulter) using a modified capillary cartridge for CE-MS analysis. A capillary coated with hydroxypropyl cellulose (HPC) was prepared according to the published method[25] from a bare fused silica capillary (50 μm inner diameter (I.D.), 360 μm outer diameter (O.D.), 65 cm in length) and was used as the separation capillary (SC). The bare fused silica capillary used as the modifier delivery column (MC) was 80 cm long with an I.D. of 100 μm and O.D of 360 μm. Prior to each run, the MC and the microvial at the sprayer tip were filled with catholyte and the SC was flushed with 4M urea at 40 psi for 3 min, and flushed with water for 5 mins. The SC was then filled by the sample mixture. An anolyte plug was injected by pressure to fill approximately 35 cm length of the SC. At the onset of focusing, the end of the MC was placed in the mobilizer vial and 0.2 psi pressure was applied to start delivering the mobilizer into the MC. Isoelectric focusing was performed by applying 30 kV voltage across the SC for a period of 22 minutes. After 22 min, the acidic mobilizer was introduced to fully replace the catholyte in both the MC and the microvial, thereby starting chemical mobilization. The pressure applied at the mobilizer vial was increased to 0.5 psi at 22 min. Time programs were carried out by the 32 Karat software (Beckman Coulter).

Mass analysis was carried out by either a Micromass Q-TOF 1E (Waters, Milford, MA) or a Finnigan LCQ^{DUO} ion trap mass spectrometer (Thermo Scientific, Waltham, MA). A CE-MS interface with a flow-through microvial replaced the standard ESI sources and was used to couple cIEF and MS.[237,292,293,386] For the Q-TOF

MS, the inlet cone temperature and voltage were 120 ºC and 60 V. For the ion trap MS, the inlet capillary temperature and voltage were 250 ºC and 45 V; the ion trap injection time was 80 ms. ESI voltage was turned on at 25 min. The voltage was 3.6 kV for both instruments and m/z range 1000-2000. Q-TOF was controlled by MassLynx (Waters) and the ion trap MS by XCalibur (Thermo Scientific). Data was processed by Sigmaplot 9.0 (Systat Software, CA).

5.3. Results and discussions

5.3.1. Constructing the pH Gradient Curve

Four pI markers representing five pIs were used to construct the pH gradient for the analysis. Different catholytes, anolytes and mobilizers were evaluated in previous work and no significant change in performance was observed.[393] Beckman neutral, HPC and N-CHO capillaries were tested by carrying out the same time program and, once again, no significant performance change was seen (data not shown). The HPC capillary was chosen because of the low cost and the capability of performing chemical mobilization for all major brand-named CAs. RNase A (pI 9.45), myoglobin (pI 7.35, 6.9), β-lac (pI 5.1) and CCK (pI 3.6) mixture with Fluka ampholytes and 30% glycerol was tested by cIEF-MS with chemical mobilization. Figure 1a shows the smoothed base peak electropherogram and peaks are identified based on mass spectra, labeled with marker names and pI values. Note that the third peak from myoglobin is likely a degradation product.[393] A pI *vs*. migration time plot shown in Figure 1b was also constructed, based on the electropherogram, and the linearity is shown to be

acceptable (r^2=0.9969) for cIEF experiments employing chemical mobilization. It also reveals that during the chemical mobilization, the pH gradient is maintained reasonably well. It should be noted that the lower pH section of the pH calibration curve is not as linear as the higher pH part.

The deviation from linear relationship in the rate of mobilization of these pH markers could be caused by the amount of protons needed to change the local pH, because the pH is 10 base logarithm of the concentration of protons. The pH gradient may not be linear, and the migration rates of the individual peaks may vary during the mobilization process. Therefore, a linear pH calibration curve may not always be accurate, and more finely spaced marker system could provide more precise pI information, especially if the analytes have to be detected off column, such as the case in cIEF-MS.

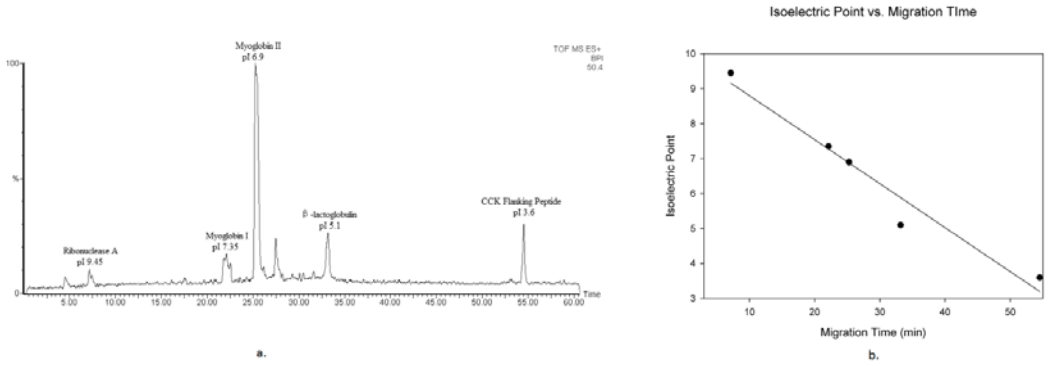

Figure 5.1. cIEF-MS Electropherogram of Different pI Markers
Four pI markers were mixed with 1% Fluka ampholytes (pH 3-10). Operation conditions are described in experimental section. 1a. Electropherogram of the pI markers and 1b. cIEF-MS of a set of pI markers vs. the migration time using a flow through micro vial CE-MS interface. cIEF-MS of a set of pI markers vs. the migration time using a flow through micro vial CE-MS interface.

5.3.2. cIEF-MS Characterization of Carrier Ampholytes

The CAs are focused along with amphoteric molecules during the focusing process. According to Thormann and coworkers' computer simulations for on-column cIEF systems, the CAs also form sharp (quasi-)Gaussian bands around their pIs. [385] The CA bands overlap with each other and form the pH gradient. Righetti *et al.* analyzed major brand CAs after gradient fractionation by CE-UV and CE-MS. The mass distributions, polydispersities and focusing properties were characterized. [394] However, there has not been a direct report on if the CAs can remain focused during the entire cIEF-MS process. In this work, we report online cIEF-MS with chemical mobilization enablingfurther characterizations of four common brand broad-range CAs: Bio-lyte, Fluka, Pharmalytes and Servalyt.

Figure 5.2 shows the cIEF-MS eletropherograms of four different types of CAs. RNaseA and CCK were added to the sample mixtures to create a reference window for chemical mobilization and to be a quality control for the focusing process. For all 4 CAs, some ampholyte peaks are detected before RNaseA, i.e. there exist species of high pIs (>9.45) in the four CAs. After CCK (pI<3.6), no peaks were detected (data not shown). This is either due to the lower positive ESI response for more acidic compounds, or due to lower abundance of those species. For Fluka and Servalyt, ampholyte peaks, which were almost baseline separated, were detected between RNaseA and CCK, and those peaks can serve as the internal pI markers with finer spacing to determine the pI of an unknown compound, which is shown in the next section.

It should be noted that the data may not represent all of the CAs present in the ampholyte mixtures because different chemical species may have different ionization efficiency, and some of the CAs, although present, may not be visible by mass spectrometry. Nonetheless, these peaks are evidences that ampholytes also form focused bands and distribute across the capillary according to their pIs, even after the chemical mobilization process. [385] To our knowledge, this proof is reported for the first time. For Bio-lyte and Pharmalytes, similar phenomena were not observed: between RNaseA and CCK, there were only elevated baselines instead of resolved peaks. This may indicate there were more ionizable species in those two types of CAs and that the peaks were too close and overlapped to be baseline separated, especially for Pharmalytes, where no distinct peaks were observed in the multi-extracted ion electropherogram. Therefore, even though Bio-lyte and Pharmalytes may provide a smoother pH gradient than Fluka and Servalyt, they cannot be used as internal pI markers unless an additional set of well defined, easily ionizable pH ladders are added. Note that the mobilization times were not the same because the interactions between different CAs and the capillary coating are different, and also the injection time/pressure and/or analysis temperature can vary slightly as well.

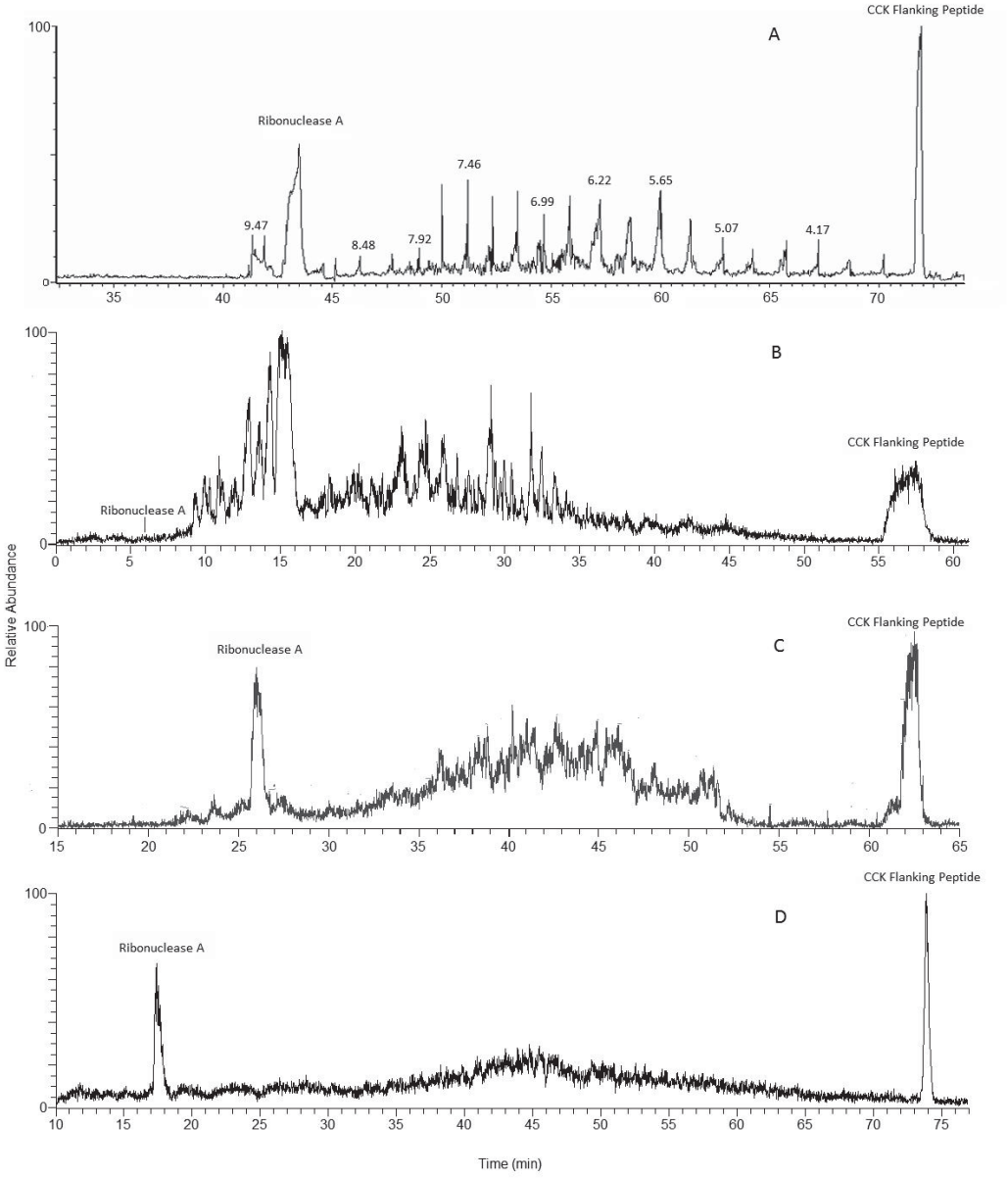

Figure 5.2. cIEF-MS Electropherograms of Common Brand Ampholytes
A, Fluka (3-10); B, Servalyt (3-10); C, Bio-lyte (3-10); D, Pharmalytes (3-10). The concentration of carrier ampholytes is 0.4% (w/v), except for Pharmalytes 1.6% (w/v). cIEF-MS conditions are provided in experimental section. For Fluka, the total ion electropherogram is provided. Tentative isoelectric point of each ampholyte peak is labeled on top of the corresponding peak. For Servalyt, Bio-lyte and Pharmalytes, multi-extracted ion electropherograms are provided. The extracted m/z ratios were chosen based on the MS spectra of common brand ampholytes.

Figure 5.3 shows MS spectra of selected individual CA peaks in each type of ampholyte investigated. All four CAs contain species with m/z over 1000, which is in agreement with the findings reported by Righetti *et al.*. [394,395] Fluka and Servalyt again show similarities with each other: peaks show up at close m/z ratios and the m/z differences between adjacent peaks are similar. Bio-lyte and Pharmalytes have similar m/z ratios for each peak and similar m/z differences; the spacing is finer between adjacent m/z peaks for Bio-lyte and Pharmalytes. Pharmalytes show no prominent peaks across the m/z range, suggesting more even mass distribution. Without further study with more real samples, it is difficult to say the implication of these data regarding to the quality and reproducibility of the pH gradients formed by these ampholytes.

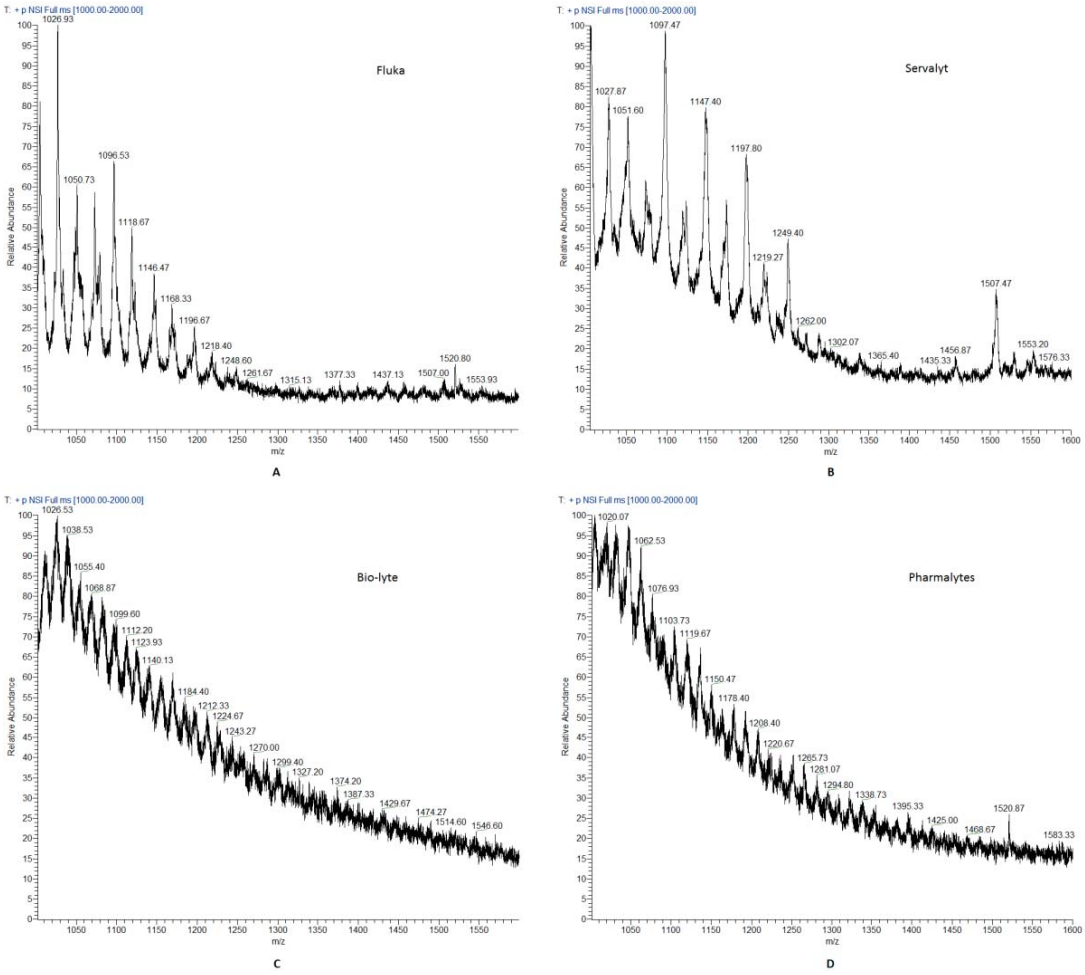

Figure 5.3. MS Spectra of Common Brand Ampholytes
A, Fluka; B, Servalyt; C, Bio-lyte; D, Pharmalytes. Experimental conditions are described in experimental section.

5.3.3. Precise pI Determination

Using the pH gradient obtained from pI markers, the pI of fine Fluka peaks can be calculated (Figure 2A). Since the spectra for different CA peaks were quite similar and no characteristic m/z for a CA appears at a specific pI, the pI values still had to be calculated based on the linear regression obtained from protein pI markers. If CAs are provided with characteristic m/z values for a designated pI, they can be the internal markers without obtaining the pH gradient from protein pI markers. The data

presented in Figure 5.4 show the potential of using the CA peaks as internal pI markers to enable more precise pI determination of amphoteric biomolecules than by using protein pI markers themselves: the smaller the spacing is, the more linear pH gradient fits. An unknown protein from the IgG sample was tested and its pI was determined by using a finer pH gradient defined by Fluka ampholytes peaks.

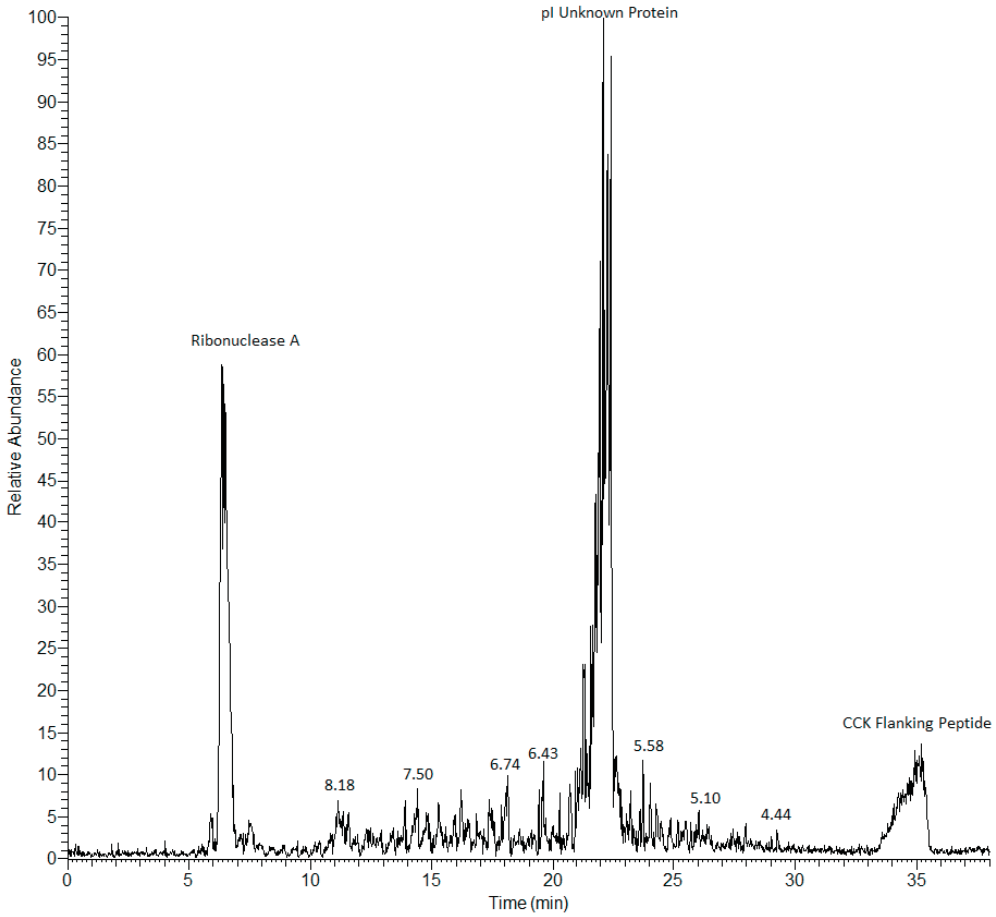

Figure 5.4. cIEF-MS Electropherogram of A pI Unknown Protein
A Protein with unknown pI is tested with 0.4% (w/v) Fluka ampholytes. cIEF-MS conditions are provided in experimental section. Tentative isoelectric point of each ampholyte peak is labeled on top of the corresponding peak. The protein mass spectrum is provided later in the chapter.

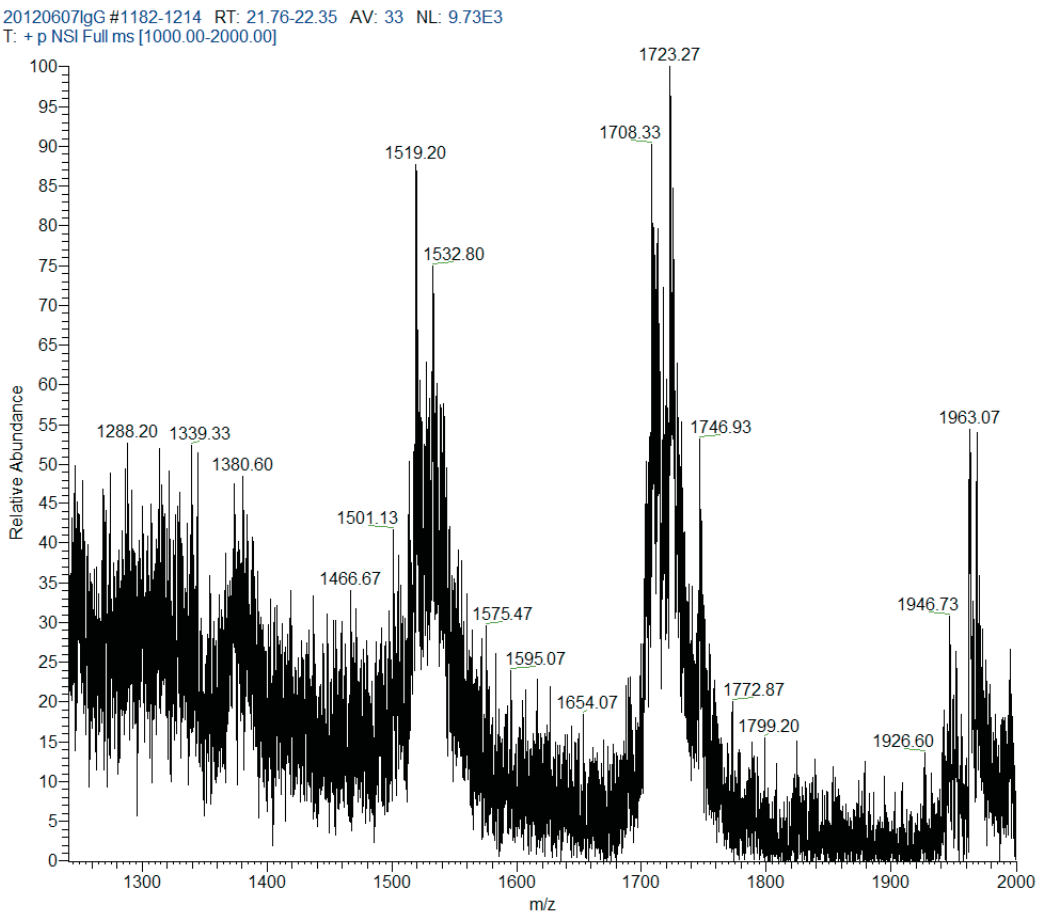

Figure 5.5. MS spectrum of the pI unknown protein

Figure 5.4 shows the total ion electropherogram for unknown protein cIEF-MS with Fluka, RNaseA and CCK. The protein mass spectrum is provided in the Figure 5. and, after deconvolution of the spectrum, the molecular mass is determined to be ~15 KD, which is not the molecular mass for a typical IgG. Due to the limitation of detection range by the mass spectrometer used, the major peaks of IgG greater than m/z 2500 were not observed. However, since the spectral pattern is similar to what has been reported at the lower m/z range for therapeutic monoclonal antibodies [396],

the protein detected could be an in-source fragment from IgG or an existing impurity. Using more finely spaced CA peaks, the pI of the unknown protein is determined to be be 6.02-6.23, centred at 6.12. Some of the ampholyte peaks no longer show up in the protein cIEF-MS, possibly due to the change in local environment for the specific pI regions by the presence of the protein, causing pH gradient distortion, which affected the focusing and distribution of CA bands. This is another important reason why using well defined ampholyte peaks, or adding a closely spaced pH ladder in conjunction with the use of these ampholytes, will enable the more precise pI determination.

5.4. Concluding remarks

The shape and distribution of focused CA bands are observed for the first time by online cIEF-MS facilitated by a novel flow-through microvial CE-MS interface. Four major brands of CAs are characterized and compared. Fluka and Servalyt share the similarity of resolved, focused ampholyte bands, and the mass spectra also show similar patterns in m/z distributions. Bio-lyte and Pharmalytes do not yield well separated ampholyte bands, which indicates a smoother pH gradient. The fine peaks of Fluka and Servalyt have the potential to serve as internal pI markers to create finer spacing for more precise pI determination of amphoteric molecules. An unknown protein sample is tested using Fluka ampholytes and the pI is determined to be 6.02-6.23, centred at 6.12.

Chapter 6. Monitoring major nutrient and metabolite concentrations in human embryonic stem cell culture using capillary electrophoresis-mass spectrometry

6.1. Introduction

Capillary electrophoresis (CE) coupled with mass spectrometry (MS) has the potential to become one of the most powerful analytical platforms because of the combination of the most powerful liquid phase and gas phase separation methods in a single process and a very sensitive and information rich detector. Much effort has been devoted to the development of high efficiency interfaces to maintain the separation of the analytes and to improve the sensitivity of MS detection.[67] As a complementary method for LC-MS and GC-MS, CE-MS can play an important role in proteomics, glycomics and metabolomics research.[269] Capabilities for analyzing proteins[155,397], carbohydrates[156,398], metabolites[157,273] and other analytes[399] have improved over the years. Metabolomics and research on small biomolecules make up a large percentage of CE-MS applications.

In molecular biology and biomedical research, mammalian cell culture processes are of increasing interest. One reason is that mammalian cells are commonly used for therapeutic protein productions in biotech industry.[400] Another reason is the promising potential to develop effective cell-based therapies for major diseases such as diabetes and cancers.[401,402] Monitoring the cell culture composition is necessary to more efficiently optimize these bioprocesses. Different analytical techniques have been used to characterize cultured mammalian cells, especially human stem cells.[403] As

cells take in nutrients and secrete metabolites during their growth, instead of analyzing the cell lysates, monitoring concentration changes of nutrients and/or metabolites in the cell culture medium can serve as a nondestructive way to provide insights into the state of the cells.

In this work, we report a CE-ESI-MS method using a flow-through microvial interface [237,238] to monitor the nutrient and/or metabolite concentration changes in the cell culture medium for human embryonic stem cell (hESCs) CA1S. Only 50 μL out of 1.5 mL conditioned medium was consumed for each analysis. An SPE procedure was used to clean up the samples before instrumental analysis. A Polyethyleneimine (PEI) coated capillary was used to speed up the separation, bringing the total completion time for the CE analysis to about 15 minutes. Selected reaction monitoring (SRM) by triple quadrupole MS identified 33 common human metabolites. The concentration variations of 25 metabolites over one culturing cycle were normalized to the concentrations in fresh medium and subsequently plotted semi-quantitatively. This method can serve as a routine procedure to monitor mammalian cell culture processes nondestructively.

6.2. Experimental section

6.2.1. Chemicals and materials

All chemicals were of analytical grade or better and were used without further purification. The standards for the metabolite, listed in Table 6.1, were obtained from Dr. David Wishart's group and were prepared as 0.2 M aqueous solution. Methionine sulfone, used as the internal standard (IS) and acetonitrile (ACN) were purchased

from Sigma-Aldrich (St. Louis, MO). Ammonia hydroxide, acetic acid (AA), formic acid (FA) and methanol (HPLC grade) were purchased from Fisher Scientific (ON, Canada). mTeSR medium was purchased from Stemcell Technologies (BC, Canada). TrypLE enzyme and PBS were obtained from Invitrogen (Grand Island, NY). BD Matrigel™ basement membrane matrix was purchased from BD Biosciences (hESC qualified, ON, Canada). All solutions were filtered by 0.22 μm membranes (Millipore Co., Billerica, MA) prior to CE analysis.

Fused silica capillary (100 μm ID, 360 μm OD) was purchased from Polymicro Technologies (Phoenix, AZ). Fused silica capillary (50 μm ID, 360 μm OD, 75 cm long) treated with a positive PEI wall coating was obtained from Beckman Coulter (Brea, CA). Six-well tissue culture plates were bought from Sarstedt (QE, Canada). Strata-X-C strong cation exchange (SCX) cartridges (30mg/1mL) were purchased from Phenomenex (Torrance, CA).

6.2.2. CA1S cell culture

The 6-well tissue culture plate was conditioned by Matrigel/H_2O (v/v 1:30) 1mL/well at room temperature for 1 hr before starting the cell culture. After aspirating the Matrigel solution, 1 mL/well mTeSR medium was then dispensed. 200,000 CA1S cells were passaged to each well on the first day of the cell culturing. The plate was kept in a CO_2 water-jacketed incubator (37 °C, 5.0% CO_2, 90% humidity) from Nuair (Tampa, FL). Approximately every 24 hours, the medium from one of the wells was collected and kept frozen at -20 °C till further analysis. The particular well was also rinsed by 1 mL PBS and followed by 1 mL TrypLE solution to detach the cells from

the plate surface. After incubated for another 10 mins, the detached cells were counted by Cedex automated cell counting system with AS20 automatic sampler (Roche, QE, Canada).

6.2.3. Sample preparation

All collected cell culture media were processed according to the following procedures before CE-MS analysis. Each individual medium was first centrifuged at 13,000 rpm for 2 mins. 50 μL supernatant was then mixed with 350 μL 0.1 M AA and 1 μL 10 M internal standard IS, and vortexed vigorously. The mixture was loaded to an SCX cartridge which was conditioned by 0.5 mL methanol and equilibrated with 0.5 mL 0.1M AA by gravity. By applying positive air pressure, sample-loaded cartridges were washed by 0.2 mL 0.1 M AA and dried. After that, analytes were eluted by 0.5 mL ammonia hydroxide/methanol (v/v 15:85). The eluates were dried by vacufuge (Eppendorf, Hamburg, Germany), and resuspended in 40 μL ACN/H_2O (v/v 2:98).

6.2.4. CE-MS

CE-MS analysis was carried out by a PA 800 plus CE system (Beckman Coulter) and an API 4000 triple quadrupole mass spectrometer (AB Sciex, ON, Canada). The turbospray ionization source was replaced by a CE-MS interface with a flow-through microvial designed by our group.[237,238] CE was controlled by the Karat 32 software (Beckman Coulter) and MS by Analyst software (AB Sciex).

Separations were carried out in the 50 μm ID PEI coated capillary (75 cm long) and another 100 μm ID fused silica capillary (90 cm long) was used to deliver the

modifier solution. Capillaries were installed in a special cartridge with temperature control for CE-MS produced by Beckman Coulter. The BGE and modifier were both methanol/H_2O/FA (v/v/v 50:49:1). Prior to each run, the separation capillary was rinsed by the BGE for 3 min with 50 psi pressure. Samples were injected hydrodynamically at 3 psi for 5 s and -30 kV was applied across the capillary during separations. The modifier solution was delivered by applying 0.3 psi pressure constantly at the modifier vial. The CE temperature was maintained 25 °C. The ESI spray voltage was +4.5 kV. MS^2 scan parameters are provided in the Table 6.1.

Table 6.1. Mass Spectrometer Parameters for Metabolites Analysis

Compound	Q1	Q3	DP	EP	CE
Glycine	76	30	35	9	17
Alanine	90	44	35	9	17
Serine	106	60	35	9	14
Creatinine	114	86	9	9	16
Dihydrouracil	115	87	59	9	16
Proline	116	70	19	9	20
Valine	118	72	62	9	14
Acetylglycine	118	43	37	9	29
Betaine	118	58	50	9	27
Cysteine	122	76	35	9	18
Nicotinic Acid	124	80	43	9	29
Pyroglutamic acid	130	84	58	9	28
L-pipecolic Acid	130	83	10	9	29
Leusine	132	86	0	9	9
isoleusine	132	86	36	9	16
Creatine	132	90	12	9	17
Asparagine	133	87	60	9	15
Aspartate	134	74	34	9	19
Homocysteine	136	90	51	9	16
Lysine	147	84	80	9	36
Glutamine	147	84	23	9	9
Glutamate	148	84	60	9	22

Compound	Q1	Q3	DP	EP	CE
Methionine	150	61	23	9	34
Histidine	156	110	18	9	18
Allantoin	159	61	34	9	15
L-Carnitine	162	103	0	9	23
Phenylalanine	166	120	75	9	17
Arginine	175	70	18	9	34
N-acetyl-L-aspartic Acid	176	74	56	9	29
L-tyrosine	182	136	56	9	17
Tryptophan	205	188	20	9	14
L-anserine	241	109	32	9	33
L-cystine	241	152	65	9	19
Glutathione	308	162	76	9	24
Methionine Sulfone	187	136	30	9	15

DP-Declustering Potential; EP-Entrance Potential; CE-Collision Energy.

6.3. Results and Discussions

6.3.1. Nutrients and metabolites analysis with PEI coated capillary

The composition of nutrients and/or metabolites are of great interest of both the metabolomics [157,273] and mammalian cell culture research communities. Understanding cell behaviors is necessary to more efficiently optimize bioprocesses. Concentration changes of nutrients/metabolites reflect cell behaviors and monitoring the concentrations in the cell culture media is nondestructive towards cells. The proper balance of these nutrients is essential for the production of recombinant proteins in genetic engineering and biopharmaceutical industry.

To analyze those positive ESI-amenable species, an acidic or neutral BGE and bare fused silica capillaries are often used. Since EOF is largely suppressed under acidic conditions, the analysis time is relatively long. An assisting pressures may be applied to speed up the separation; however, the laminar flow induced will deteriorate peak

resolutions. By using a capillary with a PEI-modified inner surface, EOF is reversed, and with the low pH BGE, the EOF mobility is high. Thus, the analysis time can be shortened to 15 mins for each run (Figure 6.1).

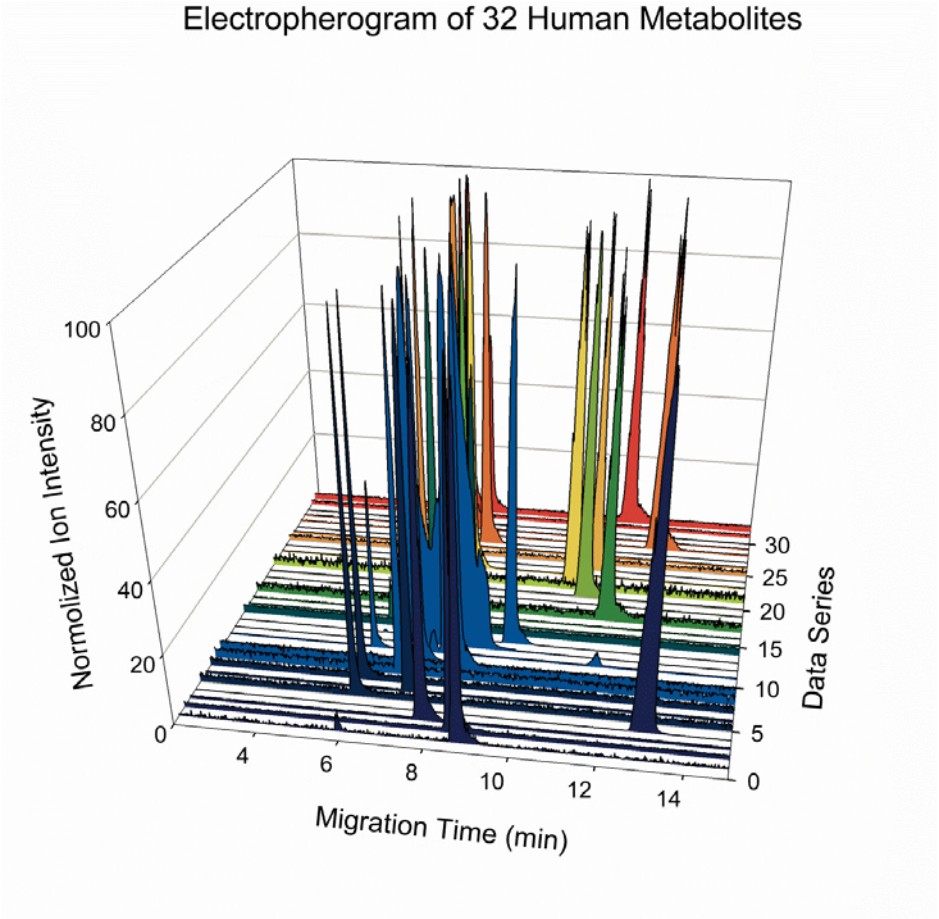

Figure 6.1. CE-MS separation for 32 common human metabolites.

The concentration of each analyte was 0.2 M in aqueous solution. Each sample was injected by 3 psi pressure for 5 seconds and separated by -30 kV voltage across the PEI coated capillary. MRM detection was conducted for every analyte. The sample sequence for 33 analytes and an internal standard is: glycine, alanine, serine, creatinine, dihydrouracil, proline, acetylglycine, betaine, valine, cysteine, nicotinic acid, pyroglutamic acid, pipecolic acid, leucine, isoleucine, creatine, asparagine, aspartate, homosystein, glutamine, glutamate, methionine, histidine, allantoin, carnitine, phenylalanine, arginine, N-acetyl-aspartic acid, tyrosine, anserine, cystine, glutathione, methionine sulfone and lysine. Peaks for every m/z ratio were normalized to the highest ion counts and plotted according to the relative ion intensity and the analyte number, as shown in Figure 6.1.

With the shorter analysis time shown in Figure 6.1, although some peaks were not fully resolved from CE, the MS/MS identification provides a second dimension of separation by m/z. Except for leucine and isoleucine, which are partially separated by CE, the other 31 analytes (glycine, alanine, serine, creatinine, dihydrouracil, proline, acetylglycine, betaine, valine, cysteine, nicotinic acid, pyroglutamic acid, pipecolic acid, creatine, asparagine, aspartate, homosystein, glutamine, glutamate, methionine, histidine, allantoin, carnitine, phenylalanine, arginine, N-acetyl-aspartic acid, tyrosine, anserine, cystine, glutathione, and lysine) are fully resolved in this analysis setup (Figure 6.1). Peaks for every m/z ratio are normalized to their respective highest ion counts and plotted against migration times.

6.3.2. Growth curve of CA1S Cells without daily medium replenishment

Figure 6.2 shows the stem cell growth curve during a four day culture cycle without daily medium replenishment. On the first day 200,000 cells were transferred to every well. After the first day, the cell numbers decreased because the passaging efficiency for CA1S stem cells was less than 100%. On each successive day, the conditioned medium from one well was collected and the cells were washed by PBS and then treated by trypLE enzyme to cut the attachments between the cells and the well wall. The intercellular connections were also broken. The cells then were counted by a Cedex automatic cell counter. Numbers of cells are plotted as a function of culturing days (Figure 6.2). From Day 2 to Day 4, the cell numbers increased almost linearly, indicating that stem cells were not in the prime reproduction phase where the number of cells should increase exponentially.[404] This phenomenon may be

caused by the starving of nutrients or it could also be due to the lack of growth factors.[405]

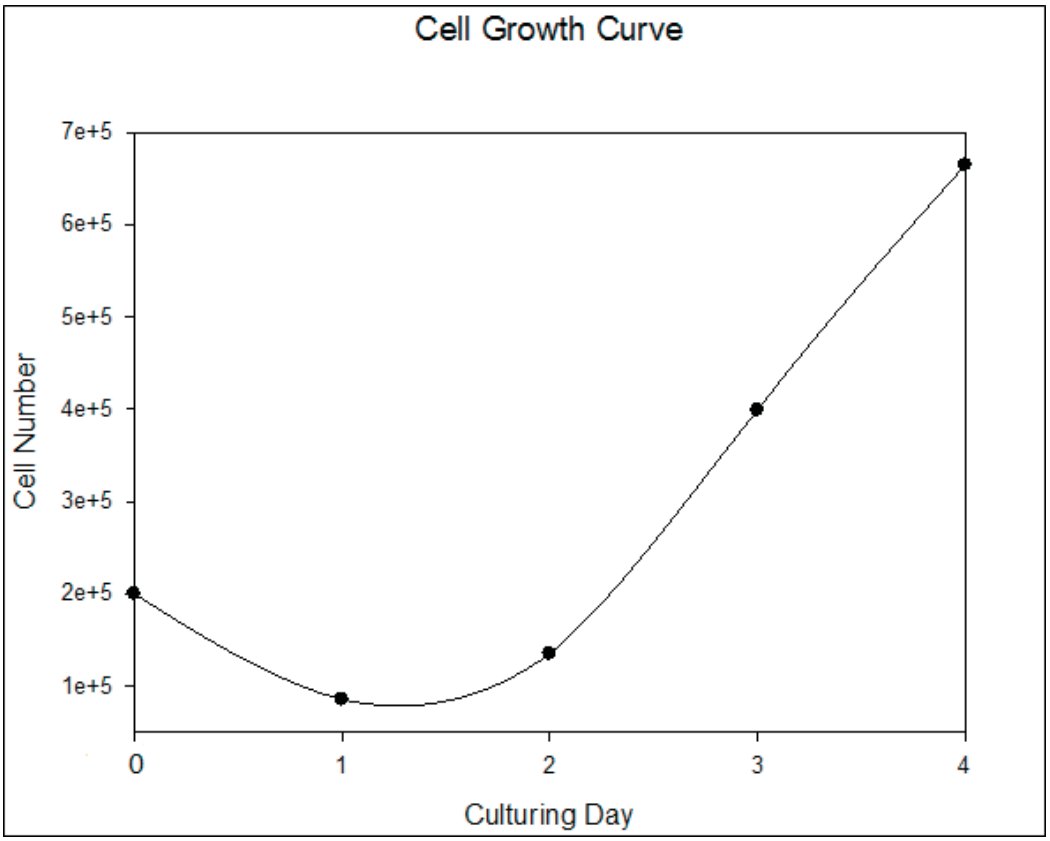

Figure 6.2. Growth curve of CA1S human embryonic stem cells in four-day starving experiment

200,000 cells/well were passaged to the 6-well tissue culture plate. In a 4-day starvation cell culture cycle, the medium in none of the culturing wells was replenished. On each successive day, the conditioned medium from one well was collected and the cells were washed by PBS and then treated by trypLE enzyme to cut the attachments between cells and well wall. The intercellular connections were also broken. Cells then were counted by a Cedex automated cell counting system with AS20 automatic sampler. Numbers of cells are plotted as a function of culturing days.

6.3.3. Consumption of nutrients monitored by CE-ESI-MS

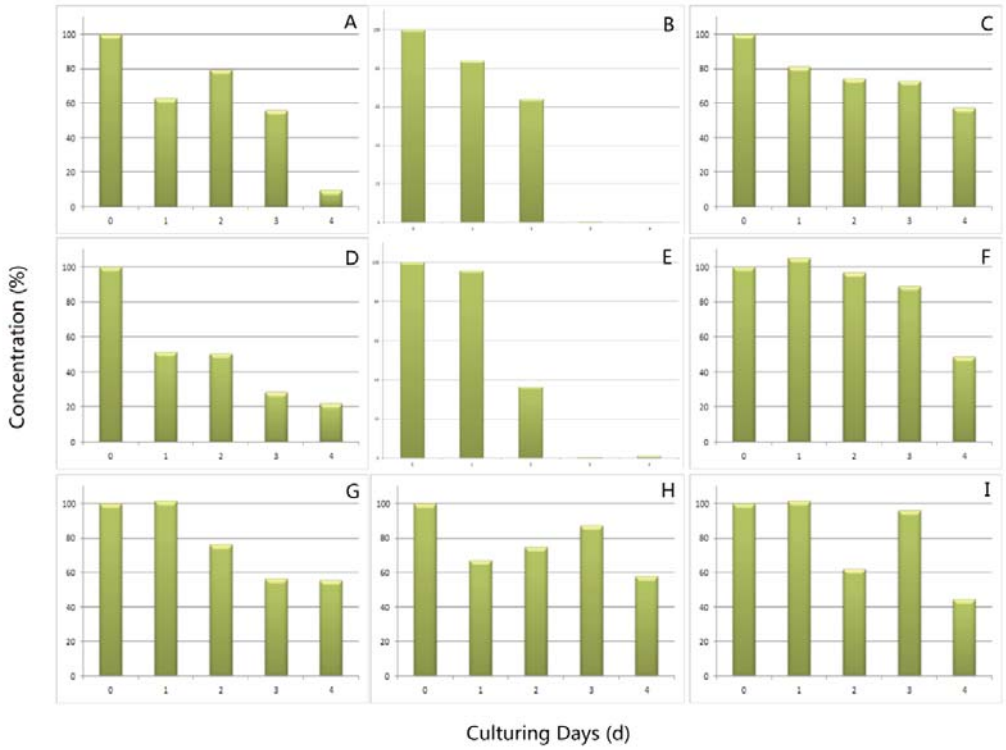

Figure 6.3. Bar charts for nutrients/metabolites that are decreasing in concentrations. A. Arginine; B. Cystine; C. Glutamate; D. Glutamine; E. Glutathione; F. Lysine; G. Phenylalanine; H. Pipecolic Acid; I. Valine. 200,000 cells/well were passaged to the 6-well tissue culture plate. In a 4-day starvation cell culture cycle, the medium in none of the culturing wells was replenished. On each experiment day, the conditioned medium from one well was collected. After SPE pretreatment, samples were analyzed by CE-ESI-MS. Concentrations were determined by peak height ratios of analytes and internal standard in MRM mode. Each ratio was then normalized to those in fresh medium and plotted as functions of culturing days. Error bars are not showing because errors are not comparable to the signals.

Figure 6.3 shows the decreasing trends for some nutrients and/or metabolites. The concentration of each analyte was determined semi-quantitatively by comparing the peak height with that of IS. All of the concentrations were normalized to the ones in

fresh mTeSR medium. As the culturing medium was not replenished during four culturing days, some analyte concentrations were observed to decrease (Figure 6.3). Those analytes are arginine, cystine, glutamate, glutamine, glutathione, lysine, phenylalanine, pipecolic acid and valine. None were observed to decrease linearly during the culturing cycle as expected, although the growth curve (Figure 6.2) showed a linear increase in cell numbers. It could be because of the sophisticated inherent biological functions of those analytes. It is also possible that with insufficient nutrients, hESCs were not under normal conditions and therefore the consumption rates were affected.

6.3.4. Increase in metabolite concentrations monitored by CE-ESI-MS

Figure 6.4 shows the increasing trends for some metabolites. The concentration of each analyte was also determined semi-quantitatively by comparing the peak heights at different days. All of the analyte concentrations were normalized to the ones in fresh mTeSR medium. During the four successive days, culturing medium was not replenished in the wells. Alanine, asparagine, aspartate, creatinine, isoleucine, methionine, nicotinic acid, pyroglutamic acid and serine concentrations were observed to increase over the culturing cycle (Figure 6.4). The observation indicates the secretion rates were larger than the intakes.

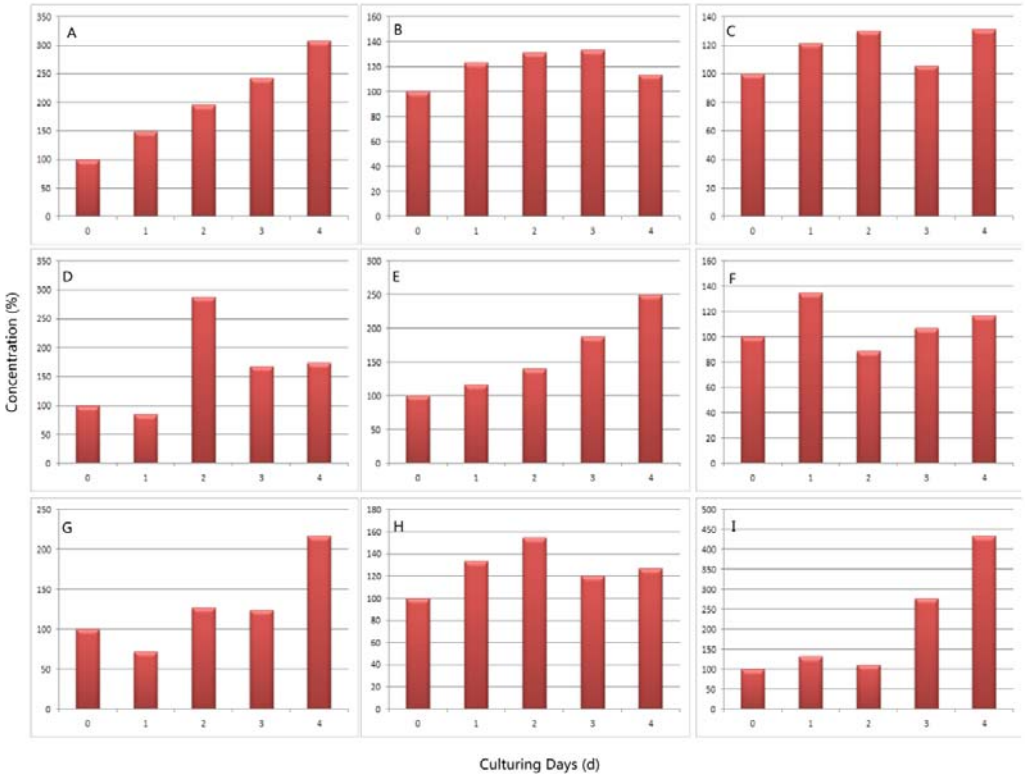

Figure 6.4. Bar charts for nutrients/metabolites in concentration increasing manners

A. Alanine; B. Asparagine; C. Aspartate; D. Creatinie; E. Isoleucine; F. Methionine; G. Nicotinic Acid; H. Pyroglutamic Acid; I. Serine. 200,000 cells/well were passaged to the 6-well tissue culture plate. In a 4-day starving cell culture cycle, the medium in none of the culturing wells was replenished. On each experiment day, the conditioned medium from one well was collected. After SPE pretreatment, samples were analyzed by CE-ESI-MS. Concentrations were determined by peak height ratios of analytes and internal standard in MRM mode. Each ratio was then normalized to those in fresh medium and plotted as functions of culturing days. Error bars are not showing because errors are not comparable to the signals.

6.3.5. Components without dramatic concentration changes in medium across the cell culture

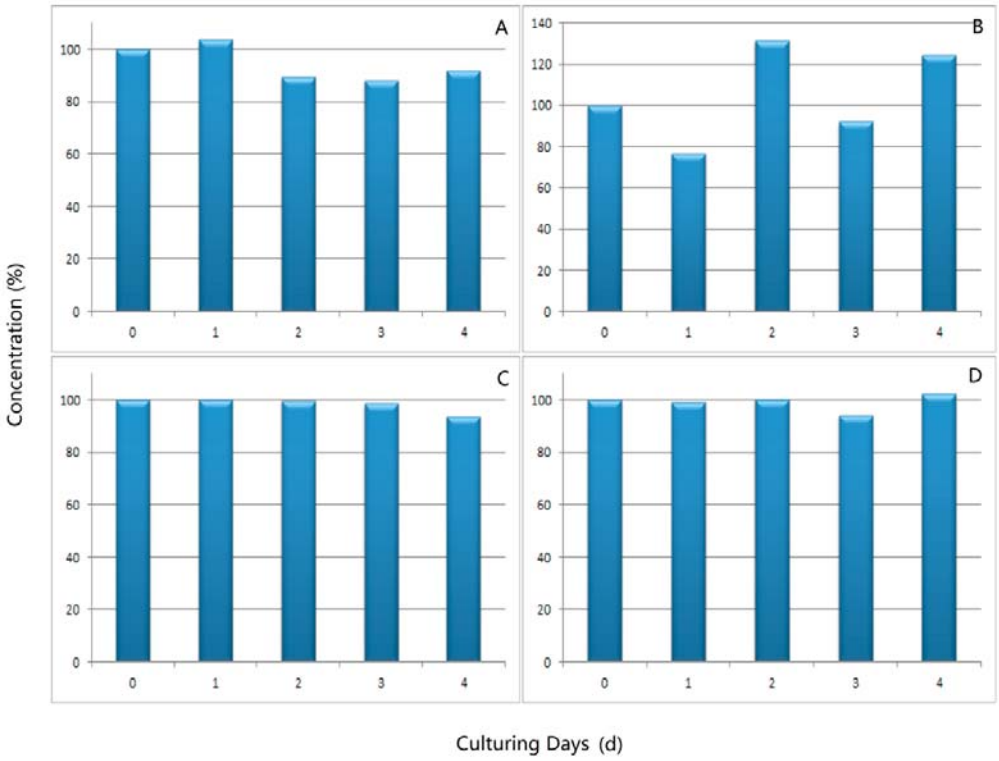

Figure 6.5. Bar charts for nutrients/metabolites without significant concentration changes over the culturing cycle
A. Cysteine; B. Leucine; C. Proline; D. Tyrosine. 200,000 cells/well were passaged to the 6-well tissue culture plate. In a 4-day starving cell culture cycle, the medium in none of the culturing wells was replenished. On each experiment day, the conditioned medium from one well was collected. After SPE pretreatment, samples were analyzed by CE-ESI-MS. Concentrations were determined by peak height ratios of analytes and internal standard in MRM mode. Each ratio was then normalized to those in fresh medium and plotted as functions of culturing days. Error bars are not showing because errors are not comparable to the signals.

In the culturing cycle, some analytes were not observed decreasing or increasing in dramatic trends (Figure 6.5 and Figure 6.6). Their concentrations stayed relatively constant without large changes over days. The semi-quantitative results are showing in Figure 6.5 and 6.6. Concentrations of cysteine, leucine, proline and tyrosine were

found to be rather stable, with concentration changing within 25% and no obvious trend (Figure 6.5). This indicates that not many cell activities involve these 4 analytes at this stage or that the intake and releasing rates are almost equal. Showing in Figure 6.6, glycine, histidine and tryptophan all displayed a peak value in the first 1 or 2 culturing days with a decrease in following days. This observation suggests that those analytes might participate in different biological activities in different phases of the stem cell starvation experiment. However, further biological experiments need to be performed to verify the reproducibility of this phenomenon.

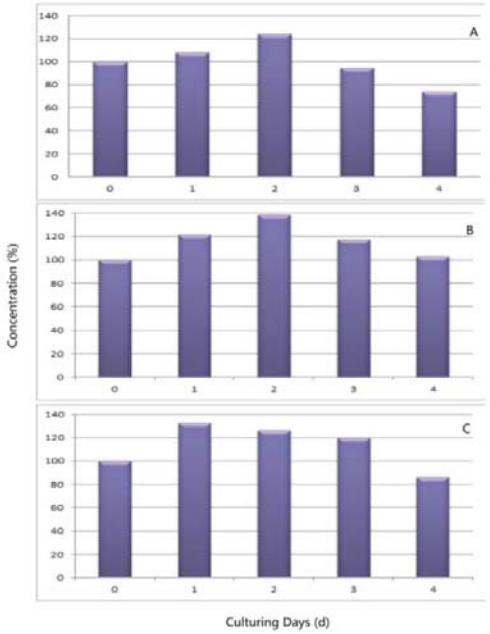

Figure 6.6. Bar charts for other nutrients/metabolites

A. Glycine; B. Histidine; C. Tryptophan. 200,000 cells/well were passaged to the 6-well tissue culture plate. In a 4-day starving cell culture cycle, the medium in none of the culturing wells was replenished. On each experiment day, the conditioned medium from one well was collected. After SPE pretreatment, samples were analyzed by CE-ESI-MS. Concentrations were determined by peak height ratios of analytes and internal standard in MRM mode. Each ratio was then normalized to those in fresh medium and plotted as functions of culturing days. Error bars are not showing because errors are not comparable to the signals.

6.4. Concluding Remarks

A relatively fast CE-MS/MS method based on PEI-coated capillary was developed to semi-quantitatively monitor the nutrient/metabolite concentration changes in cell culture media for human embryonic stem cells. Different trends in nutrient and/or metabolite concentration were grouped into three categories: consumption, secretion and relatively constant. With the short analysis time, it is possible that this highly sensitive CE-MS/MS technique will become much more widespread in future nondestructive cell culture studies.

Chapter 7. Towards better sensitivity: extending the stable operational region of a CE-ESI-MS interface by an atmospheric ion lens

7.1. Introduction

CE is a powerful tool for the analysis of biological samples because of its small sample consumption, high separation efficiency as well as the simple setup. [406] As a soft ionization technique, ESI-MS is quite popular in analyzing small quantities of biological samples. [407] Featuring versatility, ease of use and gentleness, it has been widely used to study proteins, peptides, [49] pharmaceutical drugs and their metabolites, [408] carbohydrates, [409] as well as nucleotides. [410] Coupling CE and MS through ESI could lead to the development of more powerful methods for biochemical analysis. [411,412]

However, when combining CE and ESI-MS, the mismatch between the flow rates of the two techniques needs to be addressed. [237] The net flow rate of CE can range from zero to several hundred nanoliters per minute, while most conventional ESI emitters generally operate best at flow rates above 1 μL/min. To resolve this incompatibility issue as well as achieve a complete close electrical circuit, a sheath flow liquid is usually applied to make up the mismatch, which leads to significant dilution of the CE eluent. Therefore how to successfully introduce the analyte at a lower flow rate while maintain a stable spray has always been an important topic in the research of ESI related analytical techniques. When working under low flow rate conditions, the solvent forms finer droplets that evaporate faster than those larger ones

formed at high flow rates. [413, 414] Thus, the desolvation and sampling efficiency can be improved and higher sensitivity can be achieved.

Several interfaces for CE and ESI-MS have been designed to reduce the required flow rates and therefore minimize this dilution effect. [415] In this case, the geometry of the electrospray emitter plays an important role. [154,416] Usually, the sprayer tip is a symmetrically tapered needle, [417-423] with or without a sheath flow. Some asymmetric tips also show equally good or better performance. Her *et al* developed several beveled CE-MS interfaces, [424-426] which yielded several folds of enhanced sensitivity compared to the traditional symmetrically tapered emitters. The shortcoming of these tips is that they are prone to breakage and suffer from short lifetime because they are constructed from pulled glass or fused silica capillaries. Reproducibility between batches may also be an issue. Chen group has proposed a tapered and beveled stainless steel emitter for CE-MS coupling, which displays good performance as well as robustness. [237] In this interface design, the terminal end of the separation capillary is inserted into the stainless steel needle as far as possible, so that the end of the capillary reaches the point where the diameter of the tapered inner surface is equal to the outer diameter of the capillary. A chemical modifier solution is introduced from another capillary via a tee junction. The micro vial inside the tip serves as the outlet for CE separation and the outside as the emitter for ESI.

Although the beveled tip interface has shown much better adaptability to different flow rates, [292] there is still a possibility to achieve greater stable operational regions. For this purpose, we incorporated an atmospheric pressure ion lens to the

CE-ESI-MS interface. [237,292,386] The concept of ion lens first came in the 1940s as space-charge lenses for focusing ion beams as proposed by Gabor. [427] Since then, different types of ion lenses have been used to focus ion beams under vacuum in mass spectrometers. Schneider *et al* were the first to apply ion lenses in the atmospheric pressure region of an ESI source to improve ionization and sampling efficiency [71,428,429] at reduced flow rate ranges (0.1~1.0 μL/min) with a nanospray tip. The atmospheric pressure ion lens is different from vacuum region ion lenses because the trajectories of ions follow electric field lines in the atmospheric region but not in vacuum ones, thanks to the interaction between the drag force in atmosphere and electric field force. Zhong and co-workers provided insight into the mechanism of this effect by modeling the electric field distribution in the interface setup. [430]

For the CE-ESI-MS setup, the total flow rate at the ESI emitter tip is the sum of modifier flow and electroosomotic flow (EOF). The EOF rate is determined by the conditions used in the CE separation, including the properties of the background electrolyte (BGE), the separation voltages and capillary dimensions and surface properties. The modifier solution, which is combined with the BGE at the end of the separation capillary, maintains electrical contact and increases the compatibility of the BGE with ESI. It can also be used to increase the total flow rate for more stable ESI, but this leads to increased dilution of analyte and decreased sensitivity. Therefore, expanding the range of working ranges allows greater flexibility in optimizing the separation parameters and minimizes the need for additional dilution by the modifier solution.

In the interest of evaluating the performance of the CE-ESI-MS interface with an ion lens, in this work, we compared the ESI signal intensities and stabilities without and with an ion lens under different conditions, using both direct pressure infusion and continuous electrophoresis pumping to introduce the sample for the evaluation. Based on the results of these experiments and electric field simulations, it is demonstrated that the ion lens is able to extend the operational region in both flow rate and ESI voltage dimensions. Moreover, this kind of atmospheric ion lens is not confined to coupling with this specific CE-MS interface. It could also be utilized with other types of ESI sources as long as the flow rate is within the stable working range.

7.2. Experimental section

7.2.1. Chemicals and Materials

All chemicals were of analytical grade or better and used without further purification. All amino acids were purchased from Sigma-Aldrich (St. Louis, MO). Formic acid and methanol were purchased from Fisher Scientific (Nepean, ON, Canada). A solution of water/methanol/formic acid (v/v/v 49.8:50:0.2) was used as the BGE and modifier for all infusion experiments. Arginine was dissolved in this BGE to make 25 μM and 100 μM solutions. The 25 μM arginine solution was used for the pressure infusion experiments and 100 μM for CE pumping experiments. In CE-MS experiments of amino acids, the sample solution was a mixture of serine, proline, valine, glutamine, lysine, methionine, histidine, phenylalanine, arginine and tryptophan, at concentration 100 μM each. Water/methanol/formic acid (v/v/v 49:50:1) was used as the BGE and the modifier was water/methanol/formic acid (v/v/v

49.8:50:0.2). Before use all solutions were filtered through a 0.22 µm pore size membrane (Millex, Carrigtwohill, Ireland).

Fused silica capillaries (75 and 50 µm ID, 365 µm OD) were purchased from Polymicro Technologies (Phoenix, AZ). 50 µm ID capillary whose length was 65 cm was used in all infusion experiments and 75 µm ID capillary (90 cm long) in CE pumping experiments for modifier delivery. Fused silica capillaries coated by polyethyleneimine (PEI, 50 µm ID, 365 µm OD, 65 cm long) were obtained from Beckman Coulter (Brea, CA) and used in CE pumping experiments for sample infusion.

7.2.2. Instrumentation

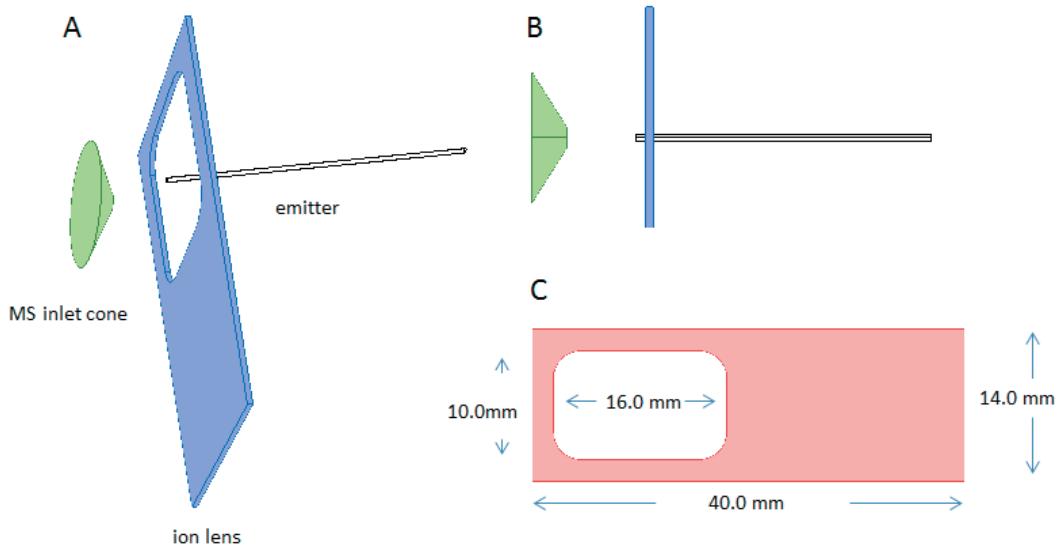

Figure 7.1. Ion Source Setup
A. Three dimensional arrangements of the emitter, ion lens and mass spectrometer (MS) inlet cone. B. Projective view of the ion source arrangement. The ion lens was set perpendicular to emitter and 1.5 mm backward from the tip end. The emitter went through the center of the ion lens oval. The emitter tip was 10 mm away from the center of MS inlet cone and was 1 mm off the central axis paralleled to the emitter. C. The dimension of the ion lens, whose thickness was 1 mm.

All experiments were performed by a Beckman Coulter P/ACE MDQ CE system with a modified capillary cartridge for CE-MS application. To carry out reproducible pressure and electroosmotic infusions, time programs were created using Beckman Coulter's 32 Karat software.

The stainless steel ion lens was made in house and the ion source setup is shown in Figure 7.1. The ion lens was made from a 40 mm×14 mm rectangular metal piece. The thickness was 1 mm. The oblong shape inside was 16 mm long and 10 mm wide. Before performing all the experiments, several potential and electric field modeling calculations were carried out by COMSOL Multiphysics (results not shown), varying the size and shape of the ion lens. Simulation results suggested that the size or shape was not essential for the modification of potential or electric field distribution. To make it easy to fabricate and mount to the interface, those parameters of the ion lens were chosen. The ion lens was arranged to be perpendicular to the emitter and 1.5 mm backward from the tip end. The emitter tip was 10 mm away from the center of the inlet cone and 1 mm deviated from the central axis. The distance between sprayer and MS inlet was optimized before all experiments and maintained the same throughout all the evaluations. A Stanford Research PS350 High Voltage DC Power Supply (Stanford Research Inc, Stanford, CA) controlled the ion lens voltage. Mass spectrometry was performed on a Micromass Q-TOF-1E mass spectrometer (Waters, Milford, MA) operating in TOF-MS mode. The standard electrospray ion source was removed and replaced by the CE-ESI-MS interface designed by our group[237]. Standard stainless steel tee unions and polyethyl ether ketone (PEEK) fittings were

purchased from Upchurch Scientific (Oak Harbor, WA). The potential of the MS inlet cone was set to be 20 V and the temperature of the source was 120 °C.

7.2.3. Infusion Methods

The sample solution was introduced through the CE capillary, while the modifier was introduced through the orthogonal port via a modifier capillary, into a flow through micro vial as described in our previous papers. [237,386] The relationship between flow rates and applied pressures was calibrated by injecting an analyte plug and measuring the migration time to MS detector under different pressures. EOF flow rate as a function of CE voltage was determined by measuring the migration time of water to the ultraviolet detector over a range of voltages. When water passed the detection window, there would be a significant baseline change for absorption. In both scenarios, linear best fits of the resulting plots were then used to calculate the actual flow rates according to the applied pressures or CE voltages. In order to elucidate the different effects of changing the sample or modifier flow rate, three different modes of infusion were tested: (i) pressure infusion with no modifier solution; (ii) constant EOF rate and varying modifier flow rates; (iii) varying EOF and constant modifier flow rate. In the pressure infusion experiments, sample infusion rates varied from 100 nL/min to 1000 nL/min over 10 steps in 2-minute intervals. When no modifier solution was added, the orthogonal port of the tee union, which is normally used to deliver a modifier solution, was blocked by a PEEK plug. The potential at the capillary inlet was set to match the electrospray voltage to avoid EOF induced by voltage difference across the capillary. In continuous CE infusion experiments, as the

capillary inner surface was coated with PEI, negative high voltage was applied at the CE inlet vial to generate EOF towards the MS inlet. In infusion mode (ii), the EOF rate was controlled at 200 nL/min and modifier flow rate varied from 0 to 1000 nL/min over 10 steps in 2-minute intervals. In infusion mode (iii), the modifier flow rate was fixed at 180 nL/min and EOF rate varied from 107 nL/min to 320 nL/min by tuning the net potential difference across the CE capillary from 10 kV to 30 kV over 5 steps in 2-minute intervals.

7.2.4. Electrospray operation

For all three aforementioned infusion modes, the electrospray voltage (V_{needle}) and ion lens voltage ($V_{ion\ lens}$) were kept constant during the flow rate variation. Averages and standard deviations of the MS signal (m/z 175) were calculated from the extracted ion intensity at m/z 175 recorded in the second minute. The same operation was repeated as the electrospray voltage increased in 0.2 kV intervals from 3.0 kV to 4.4 kV while the voltage difference between the sprayer and ion lens was held constant. Data processing was performed using MassLynx 4.0 software and Sigmaplot 9.0.

7.2.5. Potential and electric field modeling

Calculations of the electric potential and electric field strength with and without an ion lens were performed by solving the Laplace equation using COMSOL Multiphysics 3.5a software (COMSOL Inc., Los Angeles, CA). Models were built according to the measured dimensions of the emitter tip, ion lens and MS inlet, shown

in Figure 7.1. It was assumed that there was no space charge in the ion source and that the relative permittivity of the atmosphere was 1.

7.2.6. CE-MS of amino acids applying the ion lens

PEI coated capillary was used in the evaluation. Before every sample injection, the capillary was flushed by the BGE for 5 mins and the injection was realized by applying 0.5 psi pressure for 4 seconds. Separation was achieved in reverse polarity under 30 kV while the modifier flow rate was 150 nL/min. ESI voltage was 3.8 kV and MS inlet cone was 20 V. Capillary temperature was controlled at 25ºC and ion source temperature was 120ºC. When including the ion lens in tests, the voltage of it was 1.0 kV. Same experiments were repeated 5 times with and without applying the ion lens. Electropherogram was plotted. The averages and standard deviations of every peak height were calculated and listed.

7.3. Results and discussions

7.3.1. Continuous pressure infusion ESI-MS evaluation

The contour plots in Figure 7.2 show the signal/noise ratio (SNR) as a function of the electrospray voltage and the flow rate. The flow rate is determined by varying the pressure used to drive the analyte through the capillary. The stable operating range (SNR greater than 10) without an ion lens was mainly confined to electrospray voltages greater than or equal to 3.6 kV (Figure 7.2A). When proper potentials are applied on the ion lens, the lower limit of V_{needle} offering stable spray can be extended to 3.2 kV (Figure 7.2B and 7.2C). Especially for the low flow rate region (150~400 nL/min), the stable operating range can be broadened with the ion lens voltages

varying from V_{needle} – 2.6 kV to V_{needle} – 3.0 kV (Figure 7.2B, 7.2C and 7.2D). However, when the potential difference between the sprayer and the ion lens is not large enough, the stable range will be adversely affected (Figure 7.2E and 7.2F) and restricted in specific electrospray voltages and/or flow rates. The ion lens baring too high a voltage will hinder the electrospray, which can be used to turn off the electrospray during the operation by simply raising the ion lens voltages.[429]

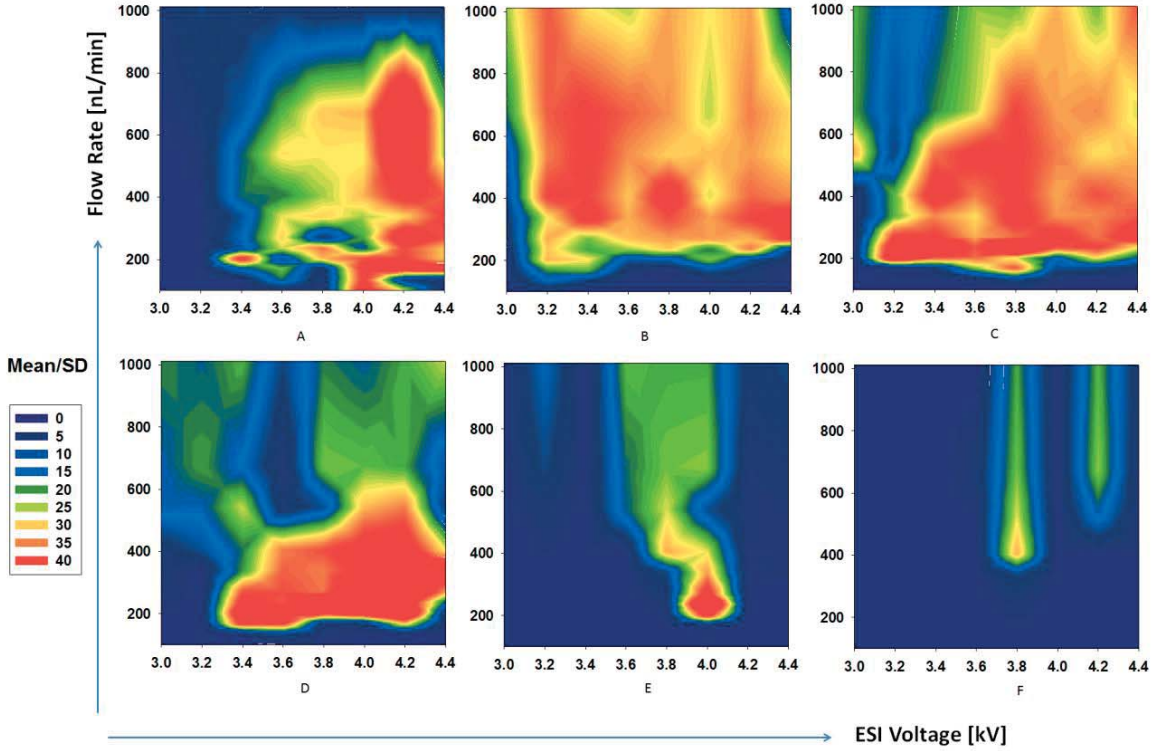

Figure 7.2. Stability Region Comparison under Different Sprayer-Ion Lens Voltage Differences by Pressure Infusion
A. Results without an ion lens. B. Results with $V_{ion\ lens} = V_{needle} – 3.0$ kV. C. Results with $V_{ion\ lens} = V_{needle} – 2.8$ kV. D. Results with $V_{ion\ lens} = V_{needle} – 2.6$ kV. E. Results with $V_{ion\ lens} = V_{needle} – 2.4$ kV. F. Results with $V_{ion\ lens} = V_{needle} – 2.2$ kV. The sample solution was 25μM arginine in the solvent of water/methanol/ formic acid (v/v/v) 49.8:50:0.2.

7.3.2. CE continuous infusion with different modifier flow rates ESI-MS evaluation

Figure 7.3 shows the signal-to-noise ratio as a function of electrospray voltage and modifier flow rate while the EOF flow rate was controlled at 200 nL/min. When the ion lens is absent, regions of stable electrospray are scattered islands in limited combinations of electrospray voltages and modifier flow rates (Figure 7.3A). To explain this phenomenon, two factors that affect the SNR of ESI are considered. On one hand, increasing the modifier flow rate can help to match up with the stable working region shown in Figure 7.2A; on the other hand, as a greater dilution effect is introduced at higher modifier flow rates, the signal average will decrease. These two contradicting effects of increasing the modifier flow rate on SNR cause the unstable working conditions. Again, it is prominent that the stable operating range is extended when an ion lens is employed. When the ion lens voltage is set to V_{needle} –3.0 kV (Figure 7.3B), in the practical operation range, all combinations of sprayer voltages and flow rates could make the electrospray stable except for the 3.4 kV – 1000 nL/min and 3.6 kV - 200 nL/min combinations. Setting the ion lens potentials equal to V_{needle} – 2.8 kV, V_{needle} – 2.6 kV and V_{needle} – 2.4 kV (Figure 7.3C, 7.3D and 7.3E, respectively) also provides satisfactory results in extending the operation regions.

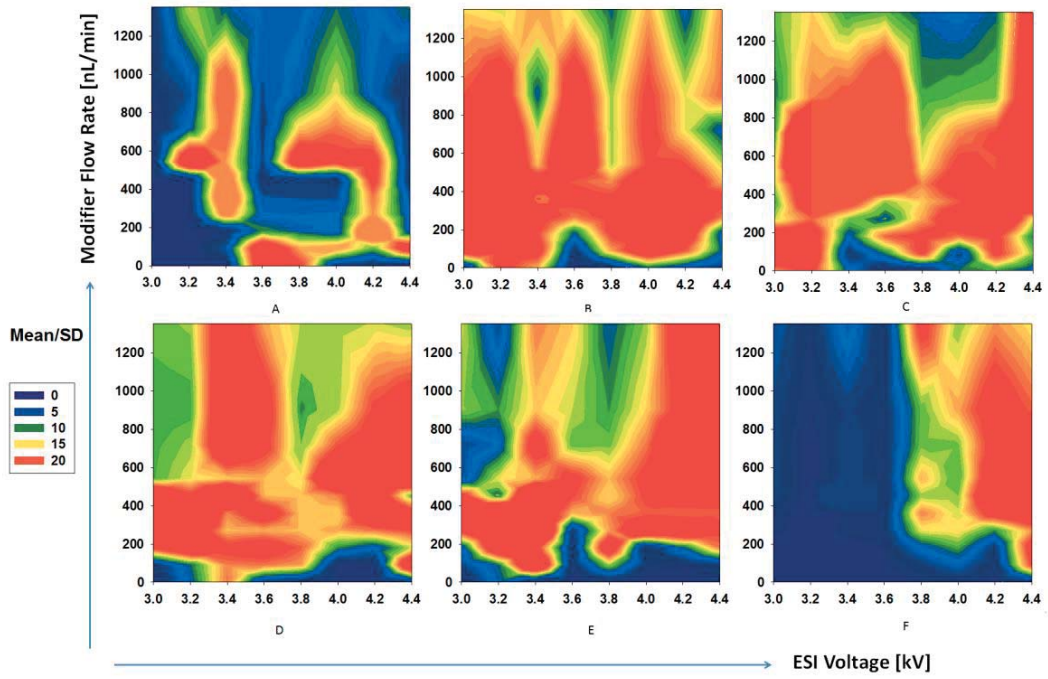

Figure 7.3. Stability Region Comparison under Various Sprayer-Ion Lens Voltage Differences by CE Continuous Infusion with Different Modifier Flow Rates
A. Results without an ion lens. B. Results with $V_{ion\ lens} = V_{needle} - 3.0$ kV. C. Results with $V_{ion\ lens} = V_{needle} - 2.8$ kV. D. Results with $V_{ion\ lens} = V_{needle} - 2.6$ kV. E. Results with $V_{ion\ lens} = V_{needle} - 2.4$ kV. F. Results with $V_{ion\ lens} = V_{needle} - 2.2$ kV. The sample was 100μM arginine dissolved in water/methanol/ formic acid (v/v/v) 49.8:50:0.2. The EOF flow rate was controlled to be 200 nL/min and the modifier solution was the same solvent as sample solution.

7.3.3. EOF flow rates evaluation

The contour plots in Figure 7.4 show the effect of electrospray voltages and EOF rates on signal-to-noise ratio. Without an ion lens, the zone of stable operation exists only at high EOF rates from 200 nL/min to 300 nL/min, while optimal electrospray voltages are 3.4 kV and higher (Figure 7.4A). The higher EOF rate region is favored because the dilution effect is smaller at higher EOF rates when the modifier flow rate is kept constant. EOF flow rates lower than 200 nL/min didn't show satisfactory signal-to-noise ratio so in further experiments with the atmospheric ion lens, the

lowest EOF flow rate was from 100 nL/min. When the ion lens is applied, the stable operation range is enlarged significantly, especially in the low EOF rate range, as well as the low electrospray voltage region (Figure 7.4B, 7.4C, 7.4D, 7.4E). This is desirable for low EOF flow rate applications. When the voltage on the ion lens is too high (Figure 7.4F) compared with the ESI voltage, fewer stable operation conditions can be achieved. Similarly, no signal is detected if the ion lens voltage is above V_{needle} – 2.2 kV.

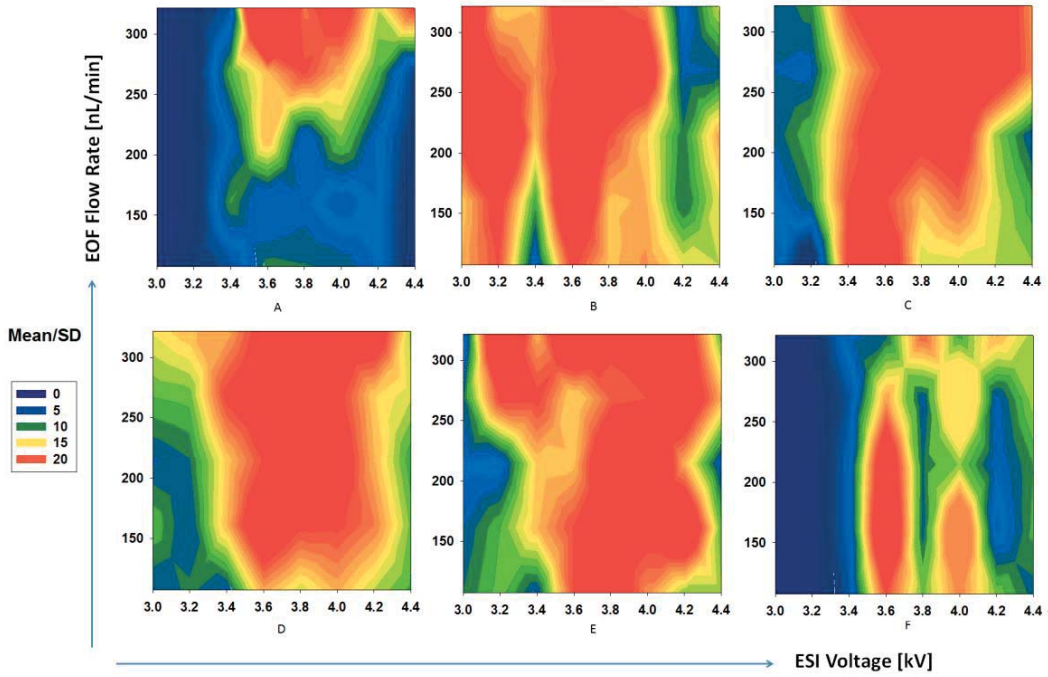

Figure 7.4. Stability Region Comparison under Various Sprayer-Ion Lens Voltage Differences by Different CE Continuous Infusion Flow Rates
A.Results without an ion lens. B. Results with $V_{ion\ lens} = V_{needle} – 3.0$ kV. C. Results with $V_{ion\ lens} = V_{needle} – 2.8$ kV. D. Results with $V_{ion\ lens} = V_{needle} – 2.6$ kV. E. Results with $V_{ion\ lens} = V_{needle} – 2.4$ kV. F. Results with $V_{ion\ lens} = V_{needle} – 2.2$ kV. The sample was 100μM arginine dissolved in water/methanol/ formic acid (v/v/v) 49.8:50:0.2.The modifier solution was the same solvent as sample solution and its flow rate was controlled to be 180 nL/min consistently.

7.3.4. Modeling of voltage distributions

Models of the electrical potential in different regions of the ion source were established according to the real dimensions of the ion source. Several comparisons in potentials between these two conditions were performed and only one is shown here due to the similar trend. The voltage of the electrospray needle was set to be 4.0 kV, MS inlet 20 V and the ion lens 1.6 kV. The reason for choosing this particular combination of voltages is because 4.0 kV is widely used as the ESI voltage in CE-MS and the 2.4 kV voltage difference between the sprayer and the ion lens has yielded a satisfactory stabilizing effect for signals in the sets of CE continuous infusion experiments (Figure 7.3 and 7.4). Figure 7.5 shows the comparison of potential and electric field line distributions in the ion source with and without an ion lens. Figure 7.5A shows the equipotential surfaces with and without the ion lens. It is clear that with an ion lens, the potential drop in the region near the sprayer tip is less severe than without. This is also demonstrated by Figure 7.5B, which shows the potential as a function of distance from the sprayer tip to the MS inlet. Whereas in the absence of the ion lens, the potential drops dramatically in all directions in the 3 mm zone of the sprayer tip. With the ion lens, the slope is much more gradual. As a result, the equipotential surfaces around the sprayer are flatter and the potential drop is mainly confined in one direction towards MS inlet. [430]

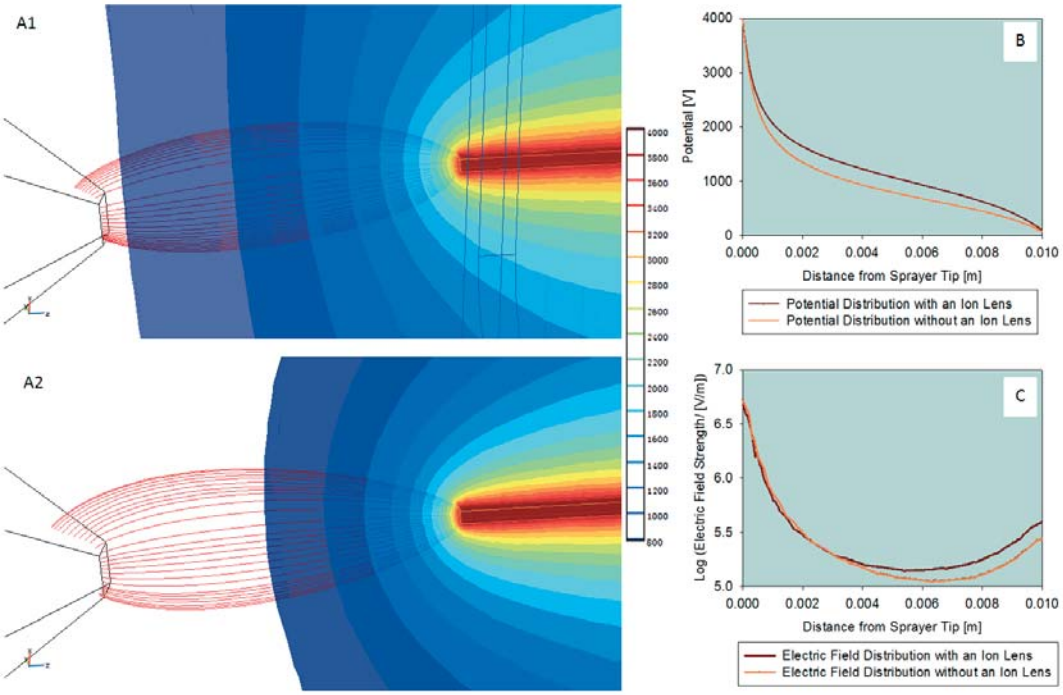

Figure 7.5. Modeling Results for Potential and Electric Field Distribution
A1. The equipotential surfaces and field lines with an ion lens. A2. The equipotential surfaces and field lines without an ion lens. B. Potentials as functions of distances from sprayer tip to MS inlet. C. Electric flied strengths as functions of distances from sprayer tip to MS inlet. Models are set as the real dimensions and voltages applied were: sprayer tip 4.0 kV, MS inlet 20 V and ion lens 1.6 kV. It is assumed there was no space charge in the ion source and the relative permittivity was set to be 1.

Another result from the flatter equipotential surfaces is that the electric field will be more convergent, which is also depicted in Figure 7.5A by the red electric field lines. These focused electric field lines will lead to more convergent trajectories for ions or ionized droplets ejected from the electrospray tip. In addition, the electric field strength that drives the ions to MS inlet is greater with an ion lens when it gets more than 3 mm away from the sprayer tip, shown in Figure 7.5C. The total energy put into a charged particle between the sprayer tip and the MS inlet is determined by the product of the net charge in the particle and the potential difference between the sprayer and MS inlet. This electric work is more evenly pumped into the charged

particle when the changing rate of potential is smaller, since the particles will experience a more constant attraction. In contrast, a burst of energy input at the beginning and letting them go freely afterwards without an ion lens will be less efficient to deliver the particles or ions to MS inlet.

As shown in Figures 7.2, 7.3 and 7.4, the operation at lower flow rates is more adaptable with the electric field generated in the ion source with an ion lens. As the initial droplet size is smaller in low flow rate operations, singly charged ions are formed faster and earlier on their way to MS inlet as the desolvation process happens faster. If single ions form too early, it is difficult for them to travel a long distance at atmospheric pressure due to the drag force of the ambient air. In this case, when the flow rate is relatively low, the field generated with an ion lens will improve the delivery efficiency because the stronger field near the MS inlet overcomes the drag force and is in more convergent directions. Furthermore, when operating at higher flow rates, the larger initial droplets need more collision energy for desolvation. With the existence of the ion lens, the stronger field intensity at 3 mm away from the tip (Figure 7.5C) accelerates the charged species faster and more electric work is converted to collision energy used for the solvent evaporation.

7.3.5. Calculation results for electric fields

The same simulation models were set up as in Section 7.3.4. All the combinations of sprayer voltage and ion lens voltage in Figure 7.2 to 7.4 were used as boundary conditions in the modeling. Maximum field strengths at the sprayer tip are shown in Figure 7.6. The electric field strengths corresponding to the stable operation

regions in Figures 7.2, 7.3 and 7.4 are mostly above 6.43×10^6 V/m, which is the ESI onset voltage. Strong enough electric field at the tip is indispensable to pull out the droplets from the Taylor cone by overcoming the surface tension. The field strength at the tip depends on the sprayer voltage and the voltage difference between sprayer and the ion lens. Figure 7.6 also shows the maximum electric field strengths as a function of these two variables respectively. As shown in Figure 7.6, when the sprayer voltage is constant, the field strength at the tip increases linearly with $|V_{needle}-V_{ion\ lens}|$. When $|V_{needle}-V_{ion\ lens}|$ is constant, the field strength at the tip increases linearly with the sprayer voltage.

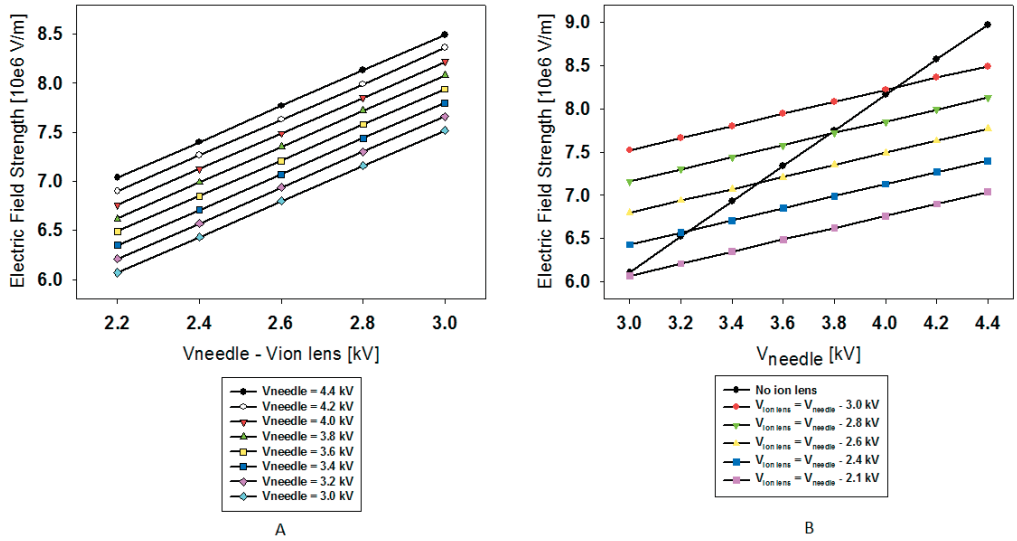

Figure 7.6. Electric Field Strengths as Functions of Sprayer Voltage and Ion Lens Voltage
A.Electric field strength as a function of the voltage differences between ESI sprayer (V_{needle}) and the ion lens ($V_{ion\ lens}$) at constant sprayer voltages. B. Electric field strength as a function of V_{needle} at constant voltage differences between V_{needle} and $V_{ion\ lens}$.

In the Figure 7.6A the electric field strength at the sprayer tip is plotted as a function of the voltage difference between the sprayer and the ion lens at different

sprayer voltages. Slopes of those curves are the same for different sprayer voltages. Only when the potential gradient (which corresponds to $|V_{needle}-V_{ion\ lens}|$) at the sprayer tip is large enough, a field strength suitable for a stable electrospray could be achieved. In Figure 7.6B the electric field strength is plotted as a function of the sprayer voltage and each curve is showing a different voltage difference between the sprayer and the ion lens. When there is an ion lens, slopes of the electric field curves are the same; without the ion lens, the field strength changes more rapidly with the sprayer voltage, illustrated by a larger slope of the function curve. For an optimized spray condition, the field strength at the tip is desired to be confined in a certain range, and then the corresponding V_{needle} range could be located according to the function curve in Figure 7.6B. It is obvious that when the slope is smaller, the applicable electrospray voltage range is wider. Thus, with proper $|V_{needle}-V_{ion\ lens}|$ value, the applicable electrospray voltage, which is the horizontal scale on the stability region plot, could be extended as demonstrated in Figure 7.2, 7.3 and 7.4.

7.3.6. Application of the atmospheric ion lens in actual CE-MS

Ten amino acids were separated with PEI coated capillary and detected by MS both without and with the ion lens, the electropherogram showing in Figure 7.7. The ion lens voltage was set 1.0 kV while the ESI voltage was 3.8 kV, so 2.8 kV voltage difference was maintained which is in accordance to the stable working condition demonstrated in Figure 7.3 and 7.4. The peak height averages and standard deviations were calculated for 5 runs each and listed in Table 7.1. As shown in the table, the signal intensities of all the ten analytes were enhanced by adding an ion lens to the

CE-MS interface, which shows the improved desolvation efficiency of ESI. Most standard deviations of peak heights were decreased except for serine which had only slight increase. That shows the stabilizing effect of the atmospheric ion lens on the ESI performance over several replicates. In total, the signal to noise ratios were improved for all the ten analytes, indicating the ion lens could be applied to a wide range of analytes in CE-MS experiments.

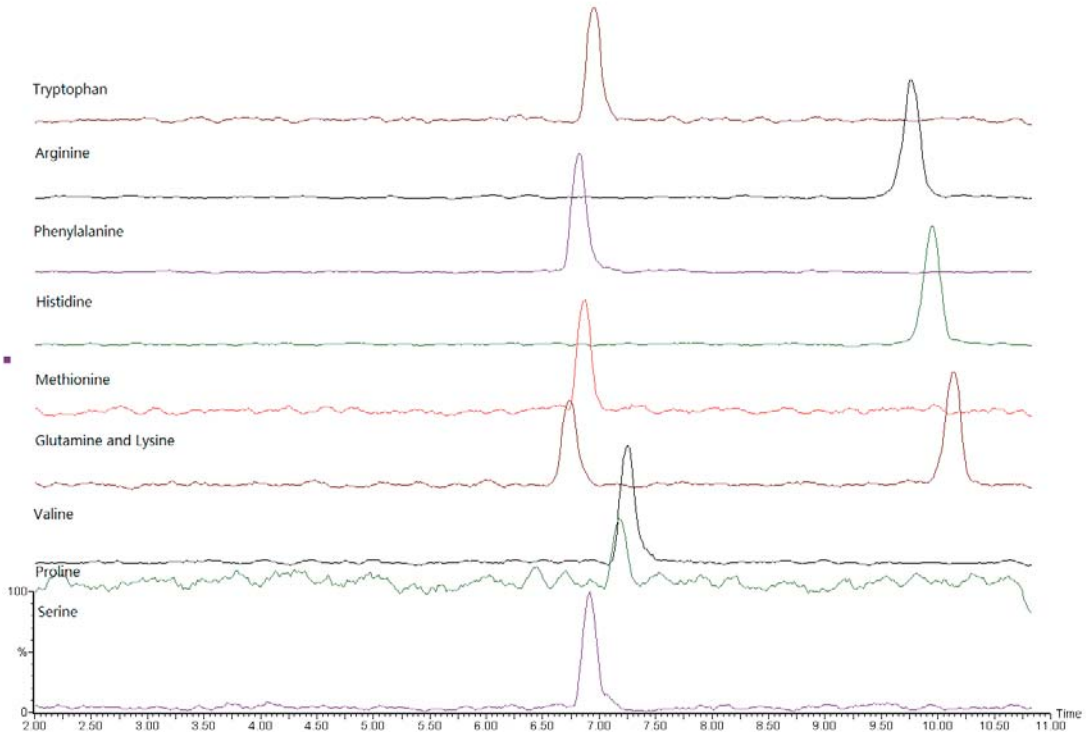

Figure 7.7. Electropherogram of 10 Amino Acids
100 μM each of serine, proline, valine, glutamine, lysine, methionine, histidine, phenylalanine, arginine and tryptophan were injected by 0.5 psi pressure for 4 seconds. The sample was separated in water/methanol/formic acid (v/v/v 49/50/1) by PEI capillary under -30 kV and detected by MS. The ESI voltage was 3.8 kV.

Table 7.1. Performance Evaluation by Multiple Analytes with and without the Ion Lens

Analytes	Intensity Average (cps)		Intensity Standard Deviation (cps^2)		Average/Standard Deviation (1/cps)	
	without	with	without	with	without	with
Serine	34.6	53.0	13.97	14.76	2.48	3.59
Proline	98.0	119.4	23.69	20.26	4.14	5.89
Valine	82.0	119.4	43.58	35.10	1.88	3.40
Glutamine	67.6	89.0	26.69	19.65	2.53	4.53
Lysine	77.6	103.4	33.15	26.10	2.34	3.96
Methionine	65.8	78.8	22.70	19.08	2.90	4.13
Histidine	104.8	124.0	57.52	31.31	1.82	3.96
Phenylalanine	175.0	205.4	69.95	55.23	2.50	3.72
Arginine	166.4	188.0	74.76	29.57	2.23	6.36
Tryptophan	81.0	111.2	42.67	22.35	1.90	4.97

The compatibility of the ion lens with different analytes was evaluated utilizing the ion lens in CE-MS experiments. Serine, proline, valine, glutamine, lysine, methionine, histidine, phenylalanine, arginine and tryptophan were separated by CE and detected by MS, both with and without the ion lens. The concentration of analytes was 100 μM of each. All CE and MS parameters were the same for the experiments in presence or absence of the ion lens. The ESI voltage was 3.8 kV and the ion lens voltage was 1.0 kV.

7.4. Concluding remarks

A stainless steel atmospheric pressure ion lens was coupled to a beveled electrospray emitter designed for CE-MS interface. Performance of ESI was evaluated by continuous pressure infusion, CE infusion with different modifier flow rates, as well as CE infusion with different EOF rates. The results showed that when there was a proper voltage difference between the ion lens and the sprayer, the extension of stable operation flow rate and sprayer voltage ranges are achieved due to the flatter equipotential surface distribution around the sprayer tip. Stronger electric field close to the MS inlet yielded by the ion lens also lead to more efficient ion transport, as

demonstrated by the modeling results from COMSOL Multiphysics. The ion lens was also applied to an actual CE-MS experiment for 10 amino acids, and comparing to the ones without the ion lens, the signal-to-noise ratios of all analytes have been increased. That suggests the ion lens is applicable to a wide range of analytes and compatible with CE-ESI-MS. Although the extension of stable working ESI voltages and flow rates was only demonstrated in positive ESI mode, similar stabilizing effect can be expected with negative ESI mode theoretically according to Laplace equation.

Chapter 8. Concluding remarks and future work

8.1. Concluding remarks

This thesis attempts to apply the flow-through microvial CE-ESI-MS interface to different separation modes and complex biological systems. To prepare an optimized online cIEF-ESI-MS system, studies are carried out to characterize the interactions among the capillary modifications, carrier ampholytes, and focusing media. A small forward consistent EOF generated from the interactions is able to ensure a good focusing and facilitate the chemical mobilization, which in turn can help a successful cIEF-ESI-MS. Feasible combinations to achieve good focusing and successful chemical mobilization are summarized. Other experiment parameters are also systematically optimized to enhance the reproducibility of cIEF process.

Using the optimized cIEF conditions, the focused carrier ampholytes bands are observed for the first time. There have been computer simulations and theoretical predictions for the cIEF process but no one has reported the directly detected carrier ampholytes bands – they are made transparent for the optical detection to eliminate the interference for target proteins. The (quasi-)Gaussian peaks of the carrier ampholytes fit the computer simulations previous carried out by other researchers and they can potentially serve as more finely spaced internal pI markers, if some synthetic information is provided by the manufacturer, to enable more accurate pI determination for amphoteric molecules. An in-source fragmentation product of IgG from rabbit serum is tested using

those carrier ampholytes bands as pI markers. The calculated pI range fits the information provided by other literature. cIEF-ESI-MS technique has also been applied to compare the proteomes of normal human embryonic stem cells and those of the starved. Preliminary results are included in the thesis, but further investigation is required.

Besides cIEF-ESI-MS, the flow-through microvial interface has also been applied to another common CE mode: CZE-ESI-MS. A rapid nutrients and metabolites monitoring method is provided in the thesis for human embryonic stem cell culture. The analysis is finished within 15 mins and 32 common nutrients and metabolites are monitored in the cell culture medium, without destroying the living cells. This method can also be applied to other cell growth monitoring.

An atmospheric ion lens is incorporated into the flow-through microvial interface to extend the stable operational regions of ESI voltage and flow rates. With the ion lens in the set-up, the stable EOF/modifier/total flow rates can be more flexible, so that the method development can be easier to begin with. This ion lens also enhances the sensitivity of CZE-MS, proved by analysis of eight amino acids. The computer calculations of the electric field distributions offer the explanation of the increased sensitivity.

8.2. Future work

8.2.1. Further optimization and application using cIEF-ESI-MS

cIEF has been widely used in the pharmaceutical industry for the characterization of protein therapeutics. In the early-stage drug development, cIEF o icIEF provides sufficient resolution and information. Also, the throughput of icIEF helps to speed up the analysis and drug development. When it comes to the late stage, not only the separation of charge variants of protein therapeutics is important, but also the identification by MS. However, current icIEF or cIEF methods are not capable to be coupled to MS online. Therefore, at the late stage of drug development, it has been the offline coupling of ion exchange chromatography, sample collection, and MS analysis, which takes up to two months for method development as well as eluent collection for minor forms.

In the thesis, the interactions between capillary modifications, carrier ampholytes, and focusing media have been studied for cIEF process and other operating parameters have also been optimized. The direct detection of focused carrier ampholytes bands shows the potential for more accurate pI determination of amphoteric molecules. For more specified target proteins, to resolve the isoforms or post-translational modifications, which slightly differ in pIs, carrier ampholytes with a narrower range can be used. For example, protein kinase B has a pI approximately at 6, so then instead of the 3-10 broad-range carrier ampholytes, the narrow-range pH 4-7 can be employed for the phosphorylation ratio determination.

The zoomed-in analysis provided by cIEF will enable the separation and analysis of protein therapeutics. The online coupling of cIEF and MS will also avoid the re-method-development as well as sample collection of ion exchange chromatography. Thus, the development time is decreased, and so is the cost of therapeutics.

Another problem with cIEF process is that proteins tend to aggregate at their pI, especially when the concentration is high, which is exactly the case after isoelectric focusing. Researchers have added urea in the focusing medium to stabilize and facilitate the dissolution of protein when optical detections (UV, LIF, etc.) are used. However, urea is not ideal for ESI-MS. So if another ESI-MS compatible protein stabilizer can be chosen, online cIEF-ESI-MS technique will lend itself wider applications in the biotechnology industry.

8.2.2. Incorporation the atmospheric ion lens into cIEF-ESI-MS system

The atmospheric ion lens has shown the enhancement of disolvation and the increase of sensitivity in CZE-MS. Since in cIEF-ESI-MS experiments, the focusing medium and carrier ampholytes also compete for the charges in the ESI process. The more convergent electric field lines offered by the atmospheric ion lens can help to increase the detection sensitivity of focused proteins. Incorporating the atmospheric ion lens into cIEF-ESI-MS system can potentially provide a good solution for the quantification of protein post-translational modifications.

8.2.3. Applying the flow-through microvial interface to other biological systems

In Chapter 6, the flow-through microvial interface has been applied to monitor the nutrients and metabolites in human embryonic stem cell culture as well as the proteome comparison between the normal cells and starved cells. Using this CE-ESI-MS method, a deeper understanding of the cell culture process can be gained. On the metabolite side, a profiling or screening can be carried out to find out the early-differentiation markers. On the protein side, an enzyme digestion can be performed before the cIEF-ESI-MS, and the profiling and database-search for the peptides can be used to identify the protein markers for the stressed cells.

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