Imaged Capillary Iso-Electric Focussing: Background, Status, and Perspectives

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Iso-electric focusing (IEF) has proven to be a very versatile separation method for the characterisation, identification, purity determination, and quantitation of amphoteric substances such as peptides and proteins. It is an indispensable and invaluable analytical tool in proteomics research, in the development of new biotherapeutics and in quality- and process-control in their manufacturing. The method is based on arranging and separating proteins according to their isoelectric points or pI, allowing to establish changes in the charge state of the molecules after structural modifications such as deamidation, oxidation, isomerisation, N-terminus modification, disulfide formation, and glycosylation. Modifications that change biochemical characteristics of biotherapeutics like their isoelectric point (pI), reduce their safety and long-term stability, influence drug efficacy, and increase undesired immunogenicity.

Nowadays, iso-electric focusing is performed in two different formats:

Slab Gel IEF

Early iso-electric focusing was executed on a flat substrate (glass or plastic plate) coated with a mixture of polyacrylamide or agarose to suppress hydrodynamic or electro-osmotic flow and so-called carrier ampholytes to establish a pH gradient. Carrier ampholytes (CA, amphoteric electrolytes) are oligomeric molecules with MW between 300-1000 obtained by the reaction of polyamines with acrylic acid (or similar structured unsaturated sulphonic acids). Each carrier ampholyte molecule, therefore, will contain several ionisable acidic and basic groups which determine the pI of each ampholyte molecule. A mixture of structurally different CA's will cover a range of pI's. The plate is flanked with trenches containing a low pH solution (anolyte) and a high pH solution (catholyte).

Laborious gel preparation with ampholyte, precise sample application, the visualisation of the separated bands, and the unstable pH gradient made this IEF method moderately reproducible and presented the user with ambiguous separation interpretations.

A significant improvement of the IEF method was obtained with the first description of Immobilised pH Gradient (IPG) gels by Bjellqvist et al [1] which makes the polymer gel itself amphoteric. Since the ampholyte molecules on an IPG are static, better reproducibility is obtained. These IPGs were introduced as a commercial product by LKB of Sweden (now GE Healthcare). The IPGs are prepared on small plates or strips the latter of which is used as the first dimension in 2D Gel Electrophoresis.

Capillary Iso-Electric Focusing (CIEF)

Despite the improvement of IEF by using prefab IPG gel plates, the method remained laborious, lacked automation, on-line detection with electronic data processing and documentation. The introduction of instrumentation for Capillary Electrophoresis (CE) followed quickly, employing IEF in a capillary. However, IEF, as described before, is a spatial separation method which takes some time for focusing to complete and sample molecules have congregated as separate bands or spots at a specific location. This in contrast to CE, which is a temporal separation method, in which the time is measured from the moment t0 when the sample molecules enter the capillary until a time t_1 when a compound passes the point of detection (PoD).

The biggest challenge in implementing IEF in a capillary, is to focus all the sample molecules in front of the point of detection during the focusing stage and once focusing has been completed, to move the focussed zones past the PoD. In addition, one must realise, that with IPG gels, the sample application is a discrete independent step after the pH gradient has been established, which in principle can take place at any physical location along the gradient on the gel plate or over the length of an IPG strip. Conventional CE sample introduction occurring at the inlet end of the separation capillary after the pH gradient has been formed is not meaningful in CIEF.

Hjertén was the first to describe IEF in a CE capillary [2] in 1985 followed by many practical and theoretical reports in peer reviewed journals and textbooks [3,4,5,6] . Despite the significant improvements in the methodology on CE-instrumentation, CIEF still requires significant user experience and expertise. The work by Mack et al [7], describing a systematic study on the usage of so-called blocking agents that confined the focussing to occur in front of the PoD, has been a major step forward in improving the method.

Principle of CIEF [8]

It is appropriate to first look into details of the CIEF process to appreciate the improvement with imaged CIEF.

For CIEF a coated fused silica (FS) capillary is used, typically 50 µm i.d. and 30-40 cm depending on the brand of the CE instrument used. The internal capillary coating (fluorocarbon, polyvinyl alcohol, DB-WAX) suppresses the electro endo-osmotic flow (EOF). At the beginning of a

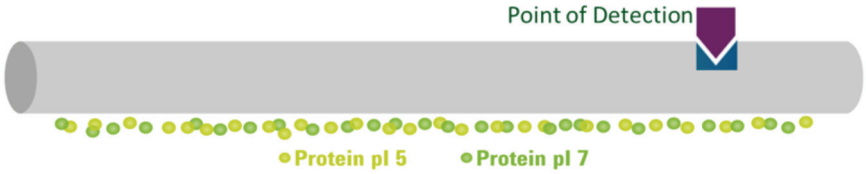

Figure 1: Depiction of the capillary composition at the beginning of a CE run. The proteins to be separated have pI 5 and 7. Carrier ampholytes, pI markers and additives are homogenously distributed over the capillary and not visualised here. (picture with permission of Agilent Technologies)

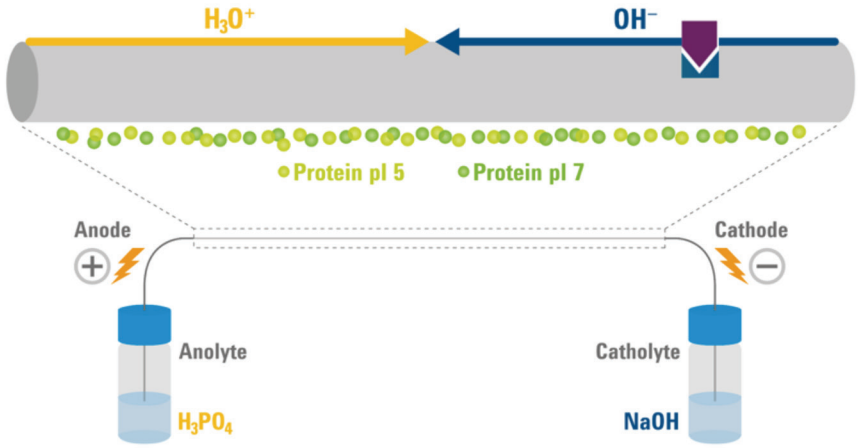

Figure 2: Schematic of initial steps of the focussing process. (Picture with permission of Agilent Technologies)

CIEF run, the whole capillary is filled with a mixture containing, carrier ampholytes, covering a specific pI range e.g. 3-8, blocking agents, the protein or peptide sample to be analysed, and pI markers. Additives may be added like methylcellulose to suppress residual EOF or urea, to improve the solubility of the focused molecules. The absence of EOF is essential in the execution of CIEF since the focussing process is disturbed by flow in the capillary.

All substances are distributed homogeneously over the whole capillary at the start. The overall pH of the sample solution is determined by the concentration and dissociation state of all substances present. The inlet vial is filled with a low pH solution (anolyte, H_3PO_4) and a positive voltage is applied here, resulting in this electrode becoming the anode. The outlet vial contains a high pH (catholyte, NaOH) solution, is grounded and the electrode at this point becomes the cathode.

This is illustrated in Figures 1 and 2.

Before the focussing process commences molecules with a low pI will have a positive charge while molecules with high pI will have a negative charge at any location in the capillary. When the voltage is switched on the situation changes as depicted in Figure 2.

When the focussing process starts, highly mobile hydrogen ions move towards the cathode, whilst hydroxyl ions move to the anode. At the same time low pI molecules which are initially positively charged move towards the cathode and negatively charged high pI molecules move to the anode. Upon migration to the electrode of opposite charge, an amphoteric molecule is protonated/deprotonated and focuses in a small zone. This is a dynamic process, with the amount of protonation and deprotonation depending on the pKa and pKb of the ionisable groups in each analyte molecule.

The completion of the focusing process in a CE instrument is monitored by the current. When all molecules are neutralised and focused, the current will have dropped significantly to a minimum level. This normally occurs after approx. 20-30 minutes. At this time, it is believed that all proteins are arranged along the capillary according to their pI.

Figure 2 shows the problem with this approach. The PoD is remote (7-10 cm depending on the CE-instrument) from the end of the capillary. Therefore, high pI proteins may become focused outside of the PoD and will be lost at the start of the mobilisation.

In a collaboration with scientists from Beckman Coulter, Vigh developed a solution [7]. So-called sacrificing ampholytes or blocking agents, which have a pI lower or higher than the pI of all sample proteins and pI markers, are introduced into the sample mixture. Iminodiacetic acid, pI 2.2 and arginine, pI 10.7 are examples. Now it must be realised that the length of a zone occupied by any ampholyte in the capillary depends on its pI, its amount in the capillary as well as the pI values and amounts of all other ampholytes in the capillary. When the concentration of the blocker is chosen properly, the focussing of analyte molecules and pI markers occurs before the point of detection. The high pI blocking agent will focus beyond the PoD. An illustration is given in Figure 3 taken from a technical note [9].

The cathodic peaks are caused by the movement of high pI sample components from the cathodic side of the capillary which passes the PoD during the focusing phase towards the anodic side. They focus before the PoD.

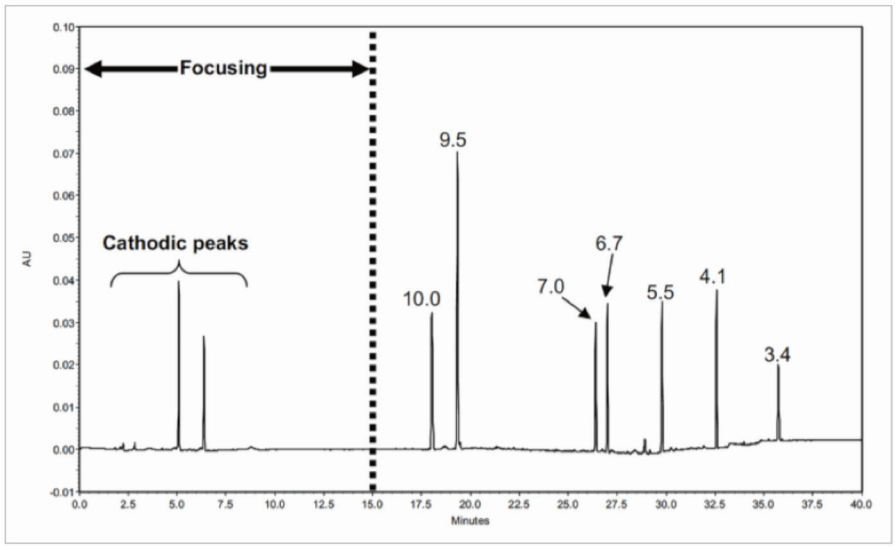

Figure 3: cIEF separation of peptide pI markers (Table 3). Sample: 200 µL of cIEF gel, 12.0 µL of Pharmalyte 3-10, 2.0 µL of each pI marker (1.25 mM), 18.0 µL of cathodic stabiliser (0.5 M Arg.) and 4.0 µL of anodic stabiliser (0.2 M IDA). Method: focusing: 15 min at 25 kV; chemical mobilisation: 25 min at 30 kV. Anolyte: 200 mM phosphoric acid. Catholyte: 300 mM sodium hydroxide. Chemical mobilising solution: 350 mM acetic acid. (Figure with permission from Sciex Separations)

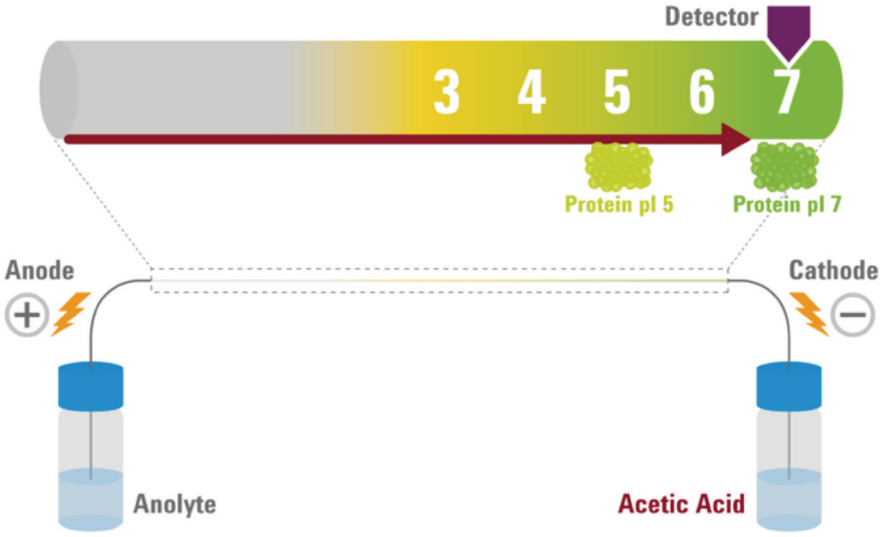

Figure 4: Principle of chemical mobilisation using acetic acid. The pH gradient is disrupted by the acid and the analytes are positively charged and move by electrophoretic force towards the cathode

As mentioned before though, all analytes are focussed before the PoD and need to be mobilised past the P0D.

The preferred mobilisation approach is to replace the catholyte vial with a vial containing acetic acid and reapply the voltage. All ampholyte molecules regain a positive charge and start to move toward the outlet vial by electrophoretic mobility as illustrated in Figure 4 (the capillary part past the detector is not shown here). The zones are detected with the built-in spectrophotometric detector (UV-DAD) at 280 nm. Since the high pI zones are closest to the PoD they are detected first. This chemical mobilisation is preferred over the pressure-driven displacement of the sample train in the capillary since significant zone broadening will occur with hydraulic flow.

The fundamental work by Vigh et al. has been commercialised by both Beckman Coulter (now Sciex Separations) and Agilent Technologies who both offer complete solutions for CIEF which has allowed the technique to become a standard methodology in the biopharmaceutical industry.

Imaged Capillary Iso-Electric Focusing (iCIEF)

The CIEF method described above has three shortcomings;

- the focussing process occurs undetected
- the focussing time is subjectively established based on the current as an indirect measurement
- the mobilisation step adds additional time to the overall run (see Figure 3).

Pawliszyn and Wu proposed to use the whole separation capillary for continuous monitoring of the focusing process which allowed visual control of the focusing process and on-column detection of the zones once focusing has been completed

Figure 5: Schematic of an iCIEF set-up (Picture courtesy of Dr Hanno Stutz, Salzburg University).

[10,11,12] . In their original work, a short, 4 cm, glass separation capillary, was used and like CIEF was connected to the anolyte and catholyte vial respectively. In a later implementation, the separation capillary was connected to an inlet and outlet capillary by a hollow fibre membrane which allows the passage of protons and hydroxyl anions into the separation capillary. This method was commercialised by Convergent Biosciences (now Protein Simple) who entered the market in 2000 with the ICE280 system, followed by Advanced Electrophoresis Solutions with their CEInfinite system in 2016. A typical sketch of this system is given in Figure 5.

The essential elements of this system are the narrow, 5 cm wide deep UV LED light source, the illuminated separation capillary, a collection lens, and a detector (camera) by which the focusing process in the separation capillary is continuously monitored. A sample mixture, as described before, fills the separation capillary completely. The sample transfer and outlet capillary are connected to the separation capillary (50 x 0.1 or 50 x 0.2 mm) by a porous hollow fibre membrane (cut-off typically 9000 Dalton). These connections are in the anolyte and catholyte vial and allow protons and hydroxyl ions to enter the separation capillary. The frames in Figure 6 clearly illustrate the focusing process. A simple sample consisting of 5 peptide pI markers are used in this example.

The first frame in Figure 6 shows a baseline of the mixture of ampholytes and 5 pI markers (peptides). Once the voltage is switched on, the marker peptides and ampholytes which are close to the anolyte vial become positively charged by protons flowing in and start to move towards the cathode. Simultaneously, the marker peptides and ampholytes close to the cathode become negatively charged by hydroxyl ions moving in and move towards the anode. Each peptide marker forms two zones which are detected as peaks moving in from the anolyte and catholyte side. It must be realised that simultaneously the ampholytes behave the same and establish the 'pH gradient' but are not detected at the wavelength (280 nm) used (frame 2 and 3).

In the 4th frame, 10 peaks from the 5 peptide markers are discerned. The small 'peaks' on the low pI side are the front of the zones of the anionic form of the marker while the high 'peaks' are the front of the zone of the cationic form of the same marker. Vice versa at the high pI side.

Frame 5 shows the focussing is coming

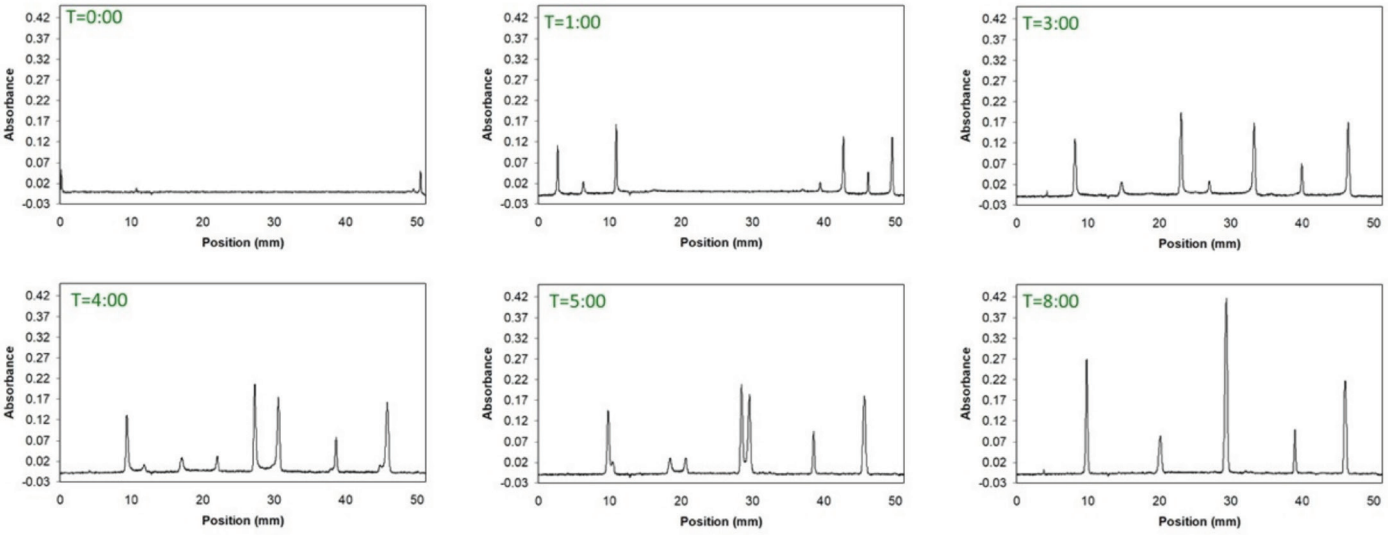

Figure 6: IEF separation process of 5 pI markers monitored in an iCIEF instrument. Frame order from left to right and from top to bottom. (frames are courtesy of Martin Donker of Isogen Lifescience, Netherlands)

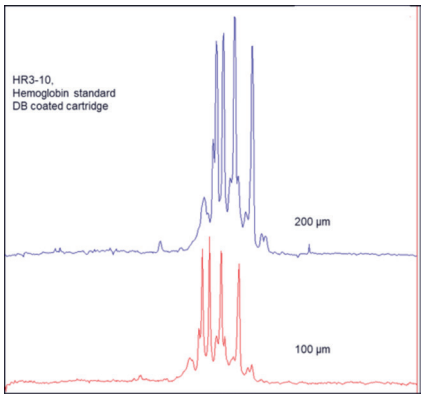

Figure 7: Separation of haemoglobin A, F, S, C isoforms. Lower trace 100 µm i.d. separation capillary, top trace 200 µm i.d. separation capillary. Sample concentration 1 mg/mL. (Figure courtesy of Tiemin Huang, Advanced Electrophoresis Solutions)

to completion with the zones at the low pI nearly and on the high pI side already focussed. In frame 6 after 8 minutes, the focusing has completed.

In a practical example, the separation of haemoglobin isoforms is shown in Figure 7.

Method development for iCIEF

Given the absence of a mobilisation step and the short length of the separation capillary, the analysis cycle time will be short compared with a conventional CIEF method.

The first step is the sample preparation containing the proteins to be analysed (approx. concentration 0.1-0.2 mg/ml), 4% ampholyte with pI's ranging from pH 3-10, and additives as required. The evolving separation is monitored continuously as illustrated in Figure 8.

This figure illustrates the benefit of continuously monitoring the development of the separation. In CIEF, only the current is taken as an indicator for completion of the focusing. But since the focussing process cannot be observed, it is arbitrary to set the proper focussing time. As shown by iCIEF, at 2.5 minutes, the current is very low and does not decrease as rapidly as earlier, but the focussing is far from complete.

With the data from the initial experiment reviewed, a narrow range pI ampholytes fitting the focussed region allows for the separation of the sample components in the same length of the separation capillary with higher resolution as illustrated in Figure 9.

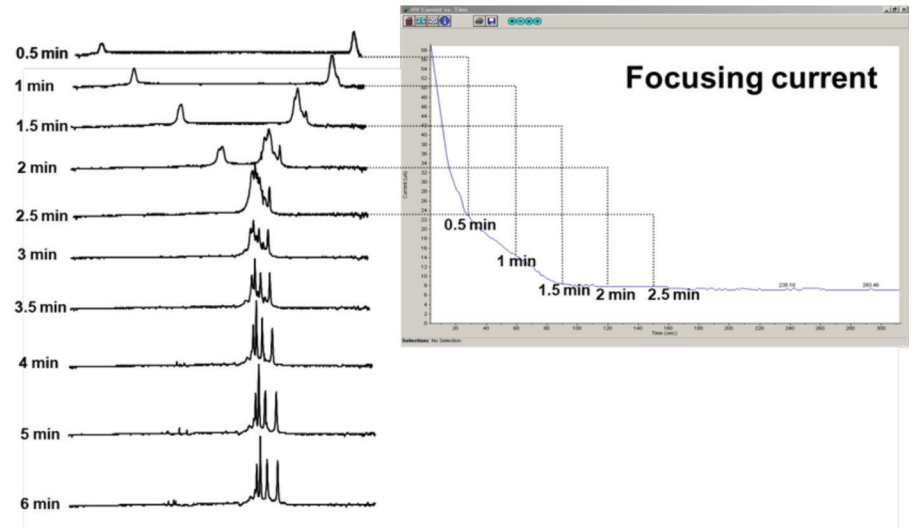

Figure 8: Sample: Hemoglobin A, F, S, C isoforms, 8% Pharmalyte 3-10, field 600 V/cm (Figure courtesy of Tiemin Huang, Advanced Electrophoresis Solutions)

Comparison CIEF and iCIEF

A comparison of both techniques is shown in Table 1 focusing on the salient features of both methods. Capillary Electrophoresis equipment offers an open platform for the execution of a diverse range of electrophoretic separations such as Capillary Zone Electrophoresis, Micellar ElectroKinetic CE, Capillary Gel Electrophoresis (esp. SDS-PAGE), Capillary Electro-Chromatography, Capillary Isotachophoresis, and CIEF. However, from a pragmatic perspective the equipment is typically allocated to a specific analysis method and so this flexibility is not always realised. Therefore, this benefit diminishes compared to using a dedicated iCIEF instrument.

iCIEF analysis cycle time (including the time needed for filling of the separation

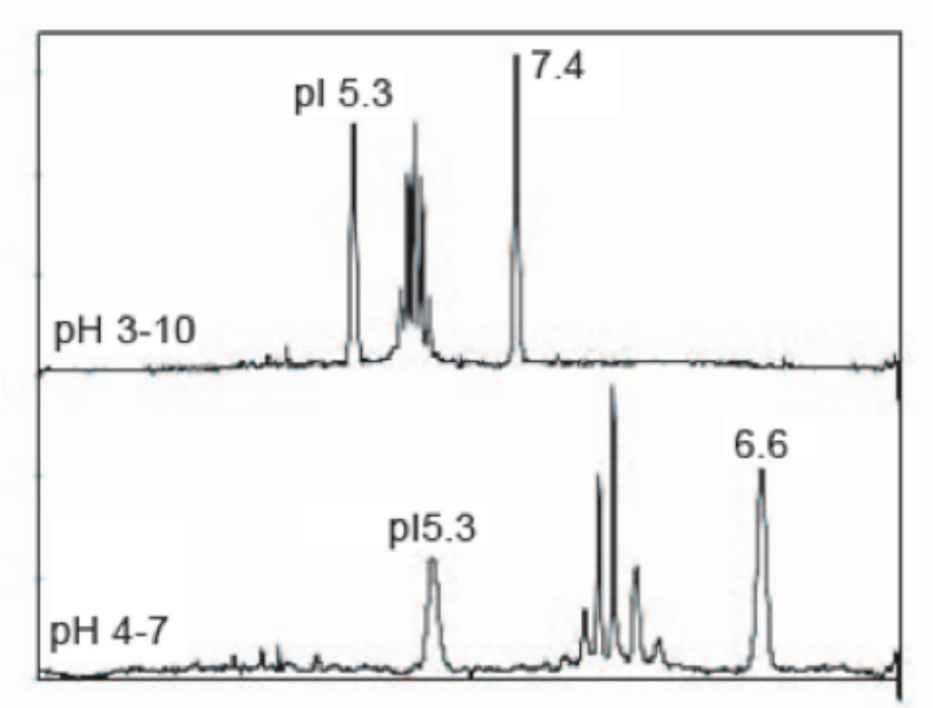

Figure 9: Effect of using smaller pI range. Sample, acidic mAB, top trace Pharmalyte 3-10, 6 mins focussing time; lower trace Servalyte 4-7, 12 mins focussing time. Anolyte 80 mM phosphoric acid, catholyte 100 mM sodium hydroxide. (Figure courtesy of Tiemin Huang, Advanced Electrophoresis Solutions)

capillary) is 5-6 times shorter than for an analysis by CIEF. Furthermore, in CIEF the result of the focussing step is only seen after mobilisation has finished whereas with iCIEF the separation is monitored as it develops. Any aberrance that may have occurred during CIEF focusing goes by unnoticed and becomes visible while mobilising. In addition, during focusing in CIEF, peaks are observed (cathodic peaks) which may raise questions about the composition of the sample under investigation.

A high level of practical competence in CE is a necessary requirement to successfully apply CIEF, whereas iCIEF is easier to use, as in most cases a standard protocol for injecting and observing the data can be followed.

The typical LODs in CIEF are 2-3x better than those obtained by iCIEF [9] since the spectrograph in CE equipment for UV-VIS detection has a stable, optimal focused optical system and highly sensitive photodiode array. The introduction of the CMOS camera as a standard detector in iCIEF has improved sensitivity, but still has some way to compete with the CIEF. Currently commercial CE and iCIEF instrumentation is rugged and robust.

There are two major providers of CE equipment (Sciex Separations and Agilent Technologies) committed to serve the market and support CIEF with consumables and application notes. For iCIEF there is one dominant manufacturer on the market (Protein Simple), although the recent introduction of the CEInfinite iCIEF system by Advanced Electrophoresis Solutions Inc. has created a more competitive landscape which will be beneficial for analytical scientists who should see improvements in the technology as the competition between the companies grows.

New developments of iCIEF

Given the ability of an iCIEF system to continuously monitor dynamic changes in the separation capillary, kinetic measurements are feasible. Eg. iCIEF can be used for determination of diffusion coefficient and hydrodynamic radius of proteins by Taylor dispersion measurement. A protein mixture is separated by IEF after which a hydrodynamic pressure is applied to move the focussed protein zone along the imaged separation channel after the high voltage is stopped. By observing the broadening of the protein zone with time, the diffusion coefficient and hydrodynamic radius can be calculated.

Alternatively, iso-electric focusing of a sample containing the protein of interest and a low molecular weight LMW ligand, one will initially see two focused zones viz the protein-ligand complex and the free protein. While continuing the focusing, one will observe that the complex zone will decrease, and the protein zone increases (see Figure 10), thus allowing KD to be calculated [14].

iCIEF/CIEF MS-Coupling

The superior resolution of CIEF and the 50-60 x concentration factor in the focussing process makes CIEF very attractive for the separation and quantitation of complex protein samples. Unfortunately, the UV detection does not allow for identity or structural information, so soon after CE-MS ESI interfacing became available, many groups have investigated coupling CIEF with MS.

In coupling CIEF/iCIEF with ESI-MS, several hurdles can be readily identified.

The zones that are mobilised from the capillary to ESI-sprayer are in nanolitre volume (4-20 nL) range, which due to the necessity of using a transfer capillary result in a loss of resolution when compared to the UV detector.

Besides the separated protein zones, the CA and other additives will become transferred to the ESI interface. This is undesirable while the ionisation process will become interfered and/or ion suppression will occur and contaminate the MS inlet capillary.

The two high voltage sources on the capillaries viz. voltage from CE and the ESI voltage need to be decoupled electrically so that there is no interference in both processes. MS systems from Agilent and Bruker have an advantage here since the ES

Table 1: Comparison CIEF and iCIEF

CAPILLARY ISO-ELECTRIC FOCUSSING	IMAGED CAPILLARY ISO-ELECTRIC FOCUSSING
Standard open platform CE Equipment: multiple purpose CE methods	Dedicated, CE system: lean system, optimised and targeted for CIEF
Long cycle time (sample introduction, focusing and mobilisation)	Fast analysis time
Long method development time	Short method development
Competence and experience required especially for troubleshooting and diagnosis	Easy to use ('black box')
Protein concentration > 0.05 mg/mL	Protein concentration >0.1 mg/mL, LOQ 1 µM [13]

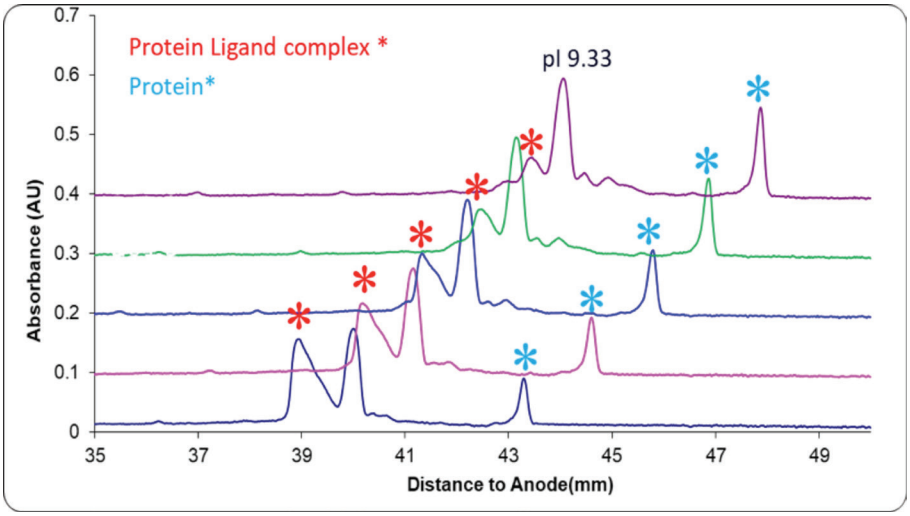

Figure 10: Time course of the dissociation of the protein-ligand complex (red asterix). The protein is marked with a blue Asterix. The ligand is not detected.

Voltage is provided from the MS with the sprayer needle set at ground voltage.

The focussing process results in a higher concentration in the focussed zone. In order to become detected in the MS, the protein must remain dissolved also in the path between the CE instrument and the ESI interface.

Many reports have been published describing solutions to overcome these challenges for CIEF-MS, unfortunately this has not led to the introduction of commercial product that supports CIEF-MS however it is beyond the scope of this paper to review these.

Advanced Electrophoresis Solutions has taken its iCIEF one step further by allowing fraction collection for further processing e.g. by MALDI-MS resp. on-line coupling with ESI-MS. A pictorial representation of the system is given in Figure 11.

The sample injection valve connects with an additional syringe pump which is filled with water or a dilute solution of methylcellulose. After sample injection and focusing has completed, this syringe delivers the contents of the iCIEF separation capillary towards a collection vial, MALDI target or via a transfer capillary to the ESI interface. Since the focusing voltage remains while pumping, focussing continues and resolution is not lost. In the transfer capillary there is no field and some zone broadening may occur. This is counteracted by using a narrow i.d. wall-coated FS capillary.

Collected protein fractions can be used for in-vial tryptic mapping followed by nanoLC-ESI MS. Recent work by Neusüß et al. demonstrated the on-line coupling by using a valve to collect fractions and direct ESI-MS or CE-MS [15].

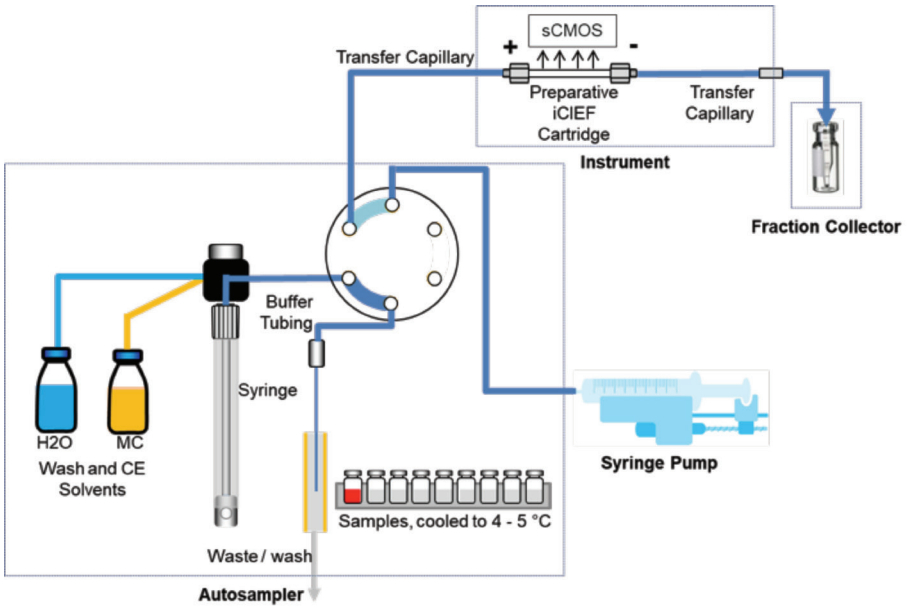

Figure 9: Pictorial representation of iCIEF fractionation system.

Conclusions and Outlook of iCIEF

With the introduction of iCIEF by the pioneers of Convergent Biosciences in the early 2000's, iso-electric focusing has become a very versatile, easy-to-use, robust and cost-effective analysis technique for protein research and biopharmaceutical development and manufacturing.

Especially in the field of proteomics and biomarker discovery or in development of manufacturing processes for HMW biopharmaceuticals, many techniques are available for determination of purity, charge heterogeneity, post-translational modfications, presence of isoforms etc. Besides CIEF, Ion-Exchange Chromatography and recently chromatofocusing are regarded alternatives for these analyses. The analyst choice for a measurement technique not only depends on the abilities and performance of an instrumental method, but also on availability of the required equipment and the levels of experienced staff in the lab.

iCIEF has been established as a routine method for protein characterisation and purity determination. The market entrance of Advanced Electrophoresis Solutions is fortunate since it will stimulate competition to the advantage of analysts and users. Moreover, AES is committed to expand the technology as a platform for iCIEF-MS and application in neighbouring fields of application

Acknowledgements

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