# 200 years of Electrophoresis

*By David Perrett*
*William Harvey Research Institute, Barts & the London School of Medicine and Dentistry, Queen Mary University of London, Charterhouse Square, London EC1M 7BQ U.K.*

This brief overview outlines the development of electrophoresis from its first observation some 200 years ago via conventional gels for macromolecule separation and capillary electrophoresis (CE) to current developments centred around lab-on-chip. By definition electrophoresis separates ionic molecules so it is ideal for the separation of simple ions to macromolecules, which are mostly ionic in nature. Most important classes of small biomolecules e.g. amino acids, nucleotides and sugars are highly charged and are easy to separate by electrophoresis. However prior to the development of CE, the application of electrophoresis was limited since it required indirect detection which was at best only semi quantitative and HPLC came to dominate their measurement. Macromolecules, such as RNA, DNA and protein, are readily separated by electrophoresis and conventional electrophoresis still dominates their separation.

## Historical background

Today electrophoresis remains a very important, if somewhat neglected, analytical technique and is now seen to have three dominant modes i.e. planar, capillary and nano separation formats. However it is just over 200 years since Ferdinand Frederic Reuss published his observations of the migration of colloidal clay particles when an electric field was applied to the solution in which they were suspended[1]. In the same experiment he also found that there was an opposite flow of water (electroosmosis) associated with the movement of the clay particles. These observations are considered to be the origins of what we now call electrophoresis. In 1816 Porret quantified the flow of water (electroosmosis) through filter paper impregnated with egg albumin. Within a few years of Reuss's observation the movement of coloured proteins, such as haemoglobin, had been observed. The early history of electrophoresis has been told by Righetti[2] and its relevance to the discovery and analysis of proteins has been reviewed by Perrett[3] .

However these early findings were little more than scientific curiosities requiring physico-chemical investigation and went under the name cataphoresis. Many laboratory suppliers sold apparatus to demonstrate these phenomena (Figure 1). The change of name came in 1909 when Michaelis suggested that the name of the technique should be changed to electrophoresis which is derived from the Greek **elektron** meaning amber (i.e. electric) and **phore** meaning bearer[4] .

The use of electrophoresis for the analysis of complex protein mixtures had to await the work of Arne Tiselius from the Karolinska in Sweden during the 1930s. Although his

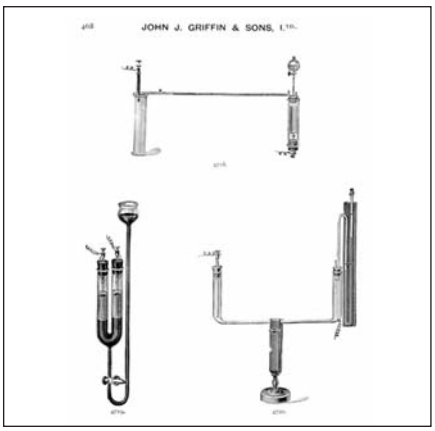


*Figure 1. Cataphoresis equipment in the Griffin Chemical Supplies Catalogue 1908*

apparatus was complex and the detection principle rather obscure, Tiselius was able to separate the five most abundant proteins that occur in human serum with relative ease and quantify their levels in both normal and some disease states. Tiselius was awarded a Nobel Prize in 1948 for his work on protein separation by electrophoresis.

From World War Two until the late 1960s the dominant separation techniques were paper chromatography for most uncharged analytes and paper electrophoresis for charged analytes such as amino acids. For both techniques indirect detection methods dominated with colour reactions being essential, e.g. ninhydrin to reveal amino acids and peptides. Electrophoresis on paper sheets followed by staining with dyes such as bromophenol blue became established for the clinical analysis of serum proteins. Paper was later replaced by more robust sheets of cellulose acetate and later nitrocellulose and Ponceau S dye became the most popular stain. Speed of separation of small ionic molecules by planar electrophoresis is limited by Joule heating and so only low voltages can be used. Elaborate high voltage systems with forced cooling were manufactured by companies such as Miles HiVolt, Shandon and Locarte in the UK (Figure 2) and Savant and Gilson in the USA. They were very large instruments that typically used 3 feet square Whatman filter paper sheets and employed 10 kV at 100 mA, a potentially lethal combination: in 1965 a graduate student at Brown University USA died from electrocution when a short circuit occurred in the high voltage electrophoresis electrical system being used.

Archer Martin and co-workers, although much better known for the development of chromatography, did much novel work on electrophoresis including using agar gels as an electrophoretic medium[5]. The use of agarose, a chemically defined variety of agar that dissolves readily in hot buffer solutions and then sets on cooling, is still commonly used for DNA separations. A major


*Figure 2. Cover of a Locarte of London High voltage electrophoresis catalogue of ca.1968. The whole system cost £898.*

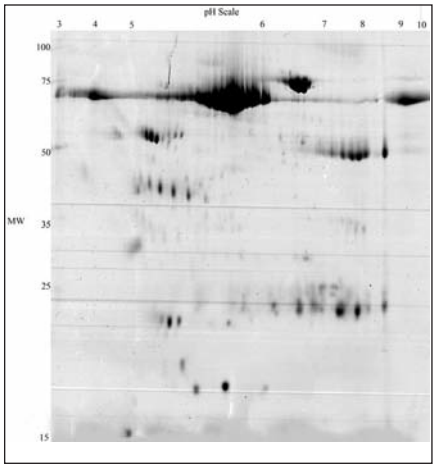


*Figure 3. 2-D (IEF-SDS-PAGE) separation of serum proteins from a patient with osteoarthritis*

improvement in the electrophoresis of proteins was the discovery of resolving power of gels made of starch in 1955[6]. Oliver Smithies (Nobel Laureate 2008), a native of Halifax, Yorkshire, remembered that his mother used starch on wash days and it set to a gel. He showed that proteins could be readily separated on slabs of starch gels and there was evidence that the separation was molecular size dependent. In 1956 Smithies[7] introduced the two dimensional separation of serum proteins: first separating them on paper followed by a starch gel. Many, still important, variants on gel electrophoresis such as immuno-electrophoresis were developed around this time. A major breakthrough occurred with the introduction of synthetic gels synthesised from polyacrylamide in 1959[8] and their use to "size" proteins in SDS-PAGE electrophoresis in 1967[9]. The use of SDS-PAGE gels in a 2-D combination with isoelectric focussing of proteins was developed by a number of groups simultaneously[3] but the credit is most often given to O'Farrell[10]. The isoelectric-focussing step was much improved with the introduction of immobilised pH gradients (IPG strips) in 1982[11] and their commercialisation in the 1990s. Although prone to many difficulties it is this 2-D approach that still forms the basis of Proteomics in many laboratories.

However electrophoresis on slab gels and similar planar formats was a qualitative technique and even the best computerised digitisation methods can only make it a poor semi quantitative methodology. So with the commercial development of first gas chromatography and then HPLC from about 1965 electrophoresis faded as an analytical method for small molecules. Gel electrophoresis continues to be used for protein analyses especially clinical diagnostic laboratories. Nucleotides and nucleosides were readily separated by paper electrophoresis but its use for the separation of nucleic acids was very limited since they have relatively constant charge to size ratio. RNA had first been separated on agar gels in 1964[12] and the still widely used background electrolyte of TRIS-borate-EDTA (TBE) was introduced in 1968[13]. The separation of DNA by gel electrophoresis had to wait a few more years and was probably first reported in 1971 by Danna and Nathans[14]. A classic paper by Gilbert and colleagues in 1974 reported the use of TBE buffers for the base specific sequencing of DNA fragments following chemical cleavage[15]. This became a standard technique in DNA sequencing until the arrival of modern sequencers in the late 1990s.

Planar separations of serum proteins on agarose gels continues to be a standard technique in many clinical laboratories. It is a skilled task and although throughput is important it is not a method needing to generate instant results nor is absolute quantitation important. Similar considerations apply to the separation of haemoglobins in the characterisation of haemoglobinopathies and thalassemias, which is usually done on cellulose acetate strips. Clinical laboratories have used semiautomatic planar electrophoresis system for some years. Such equipment has been supplied by companies such as Bio-Rad, Hoeffer etc. and high throughput fully automated clinical electrophoresis equipment is available from Sebia (France).

## Capillary Electrophoresis

The major bugbear with all electrophoresis is the generation of Joule heat with its many detrimental effects on electrophoretic separations and resolution. This was effectively overcome with the introduction of capillary electrophoresis by Jorgenson and colleagues. In three classic papers [16,17,18] published in 1981 Jim Jorgenson demonstrated that by increasing the surface to volume ratio i.e. by using a capillary, the heat generated by electrophoresis was readily lost. Very high voltages could then be employed to enable high speed analyses with on-line detection and both exceptional resolution, and enhanced sample sensitivity.

The electroosmotic flow generated by the silanol groups of the polyimide coated silica capillary's inner surface gives an efficient and fast plug flow to the electrolyte that is in contrast to the parabolic flow found in pumped LC columns and so helps generate very efficient peak shapes . CE is therefore characterised by its ability to resolve using a high applied d.c. voltage (with field strengths up to 500 V/cm), the charged components of complex aqueous samples with very high resolution (N>1 million plates/m). Unlike traditional methods CE performs with levels of analytical precision similar to HPLC but typically uses less than 10 nl of sample. By the early 1990s nearly every manufacturer of HPLC equipment had a CE system in their catalogue. Thousands of papers using CE were being published annually but the original hype from the companies and many academics was beginning to fade. Problems with migration time irreproducibility leading to poor quantitation were commonplace, sensitivities did not match even those of standard HPLC systems and surprisingly the separation of proteins was proving very difficult.

Very shortly after Jorgenson's first publications Terabe[19] introduced a novel variant on CE which enabled the separation of hydrophobic compounds by a pseudoelectrophoretic mechanism. In this variant called micellar electrokinetic chromatography (MEKC) non-polar compounds distribute themselves between an electrosmotically driven mobile phase and the charged surfactant micelles electrophoretically migrating in the opposite direction. In the commonest mode the

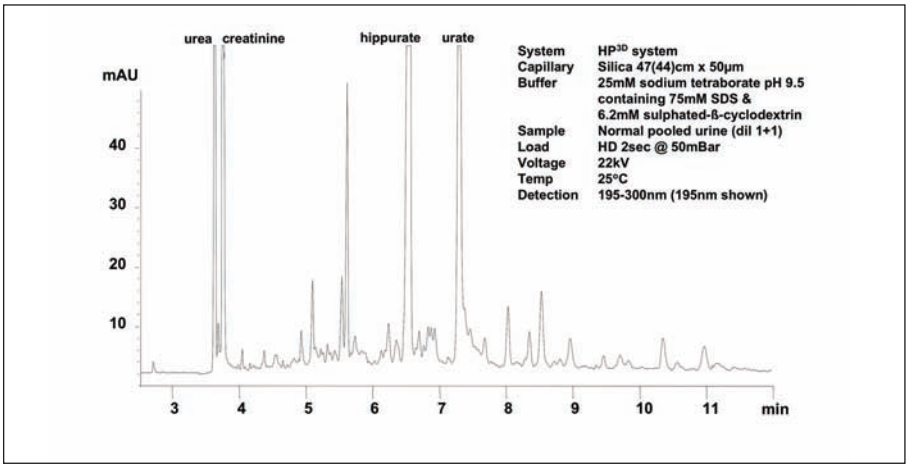


*Figure 4. U.V. absorbing compounds in human urine separated by sulphated-β-cyclodextrin modified MEKC.*

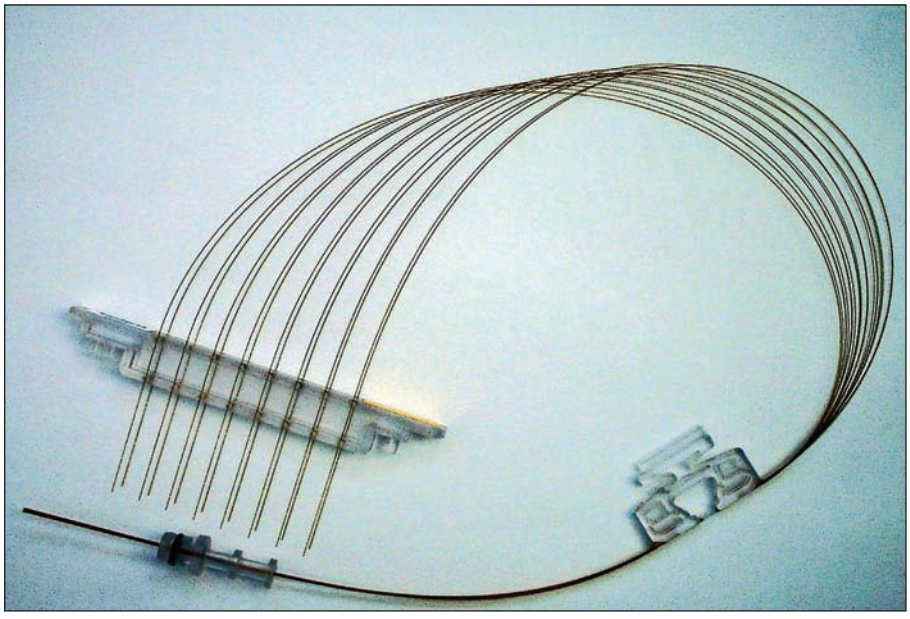

*Figure 5. Megabase multicapillary*

surfactant is the anionic detergent is sodium dodecyl sulphate at concentrations above its critical micellar concentration (ca. 8.2mM). Although the efficiency of MEKC is somewhat below that of CZE with typically N<200000 plates/m the separations can be impressive. The presence of micelles in the electrolyte does not compromise any underlying CZE separations and so complex samples containing both charged and un-charged molecules can be analysed with ease. This has been used to good effect in metabolomic screening of human samples [20,21] and is illustrated in (Figure 4).

By the start of the new millennium only two manufacturers, Agilent and Beckman, were still actively marketing analytical CE systems into small niche markets such as chiral analyses in the pharmaceutical industry and high resolution analyses in the biomedical area e.g. for metabolomic studies. A fresh period of hyperactivity followed from 1995 when exceptional separations of pharmaceuticals using capillary electrochromatography (CEC) were published. In this technique the capillary was packed with an appropriate HPLC packing material and the eluent was driven down the capillary using the generated EOF. All this could be achieved using only slightly modified versions of the available CE systems. However CEC proved even less reliable in practice then CE itself and few workers have continued to use the technique.

Around 2000 this author would often ask analytical colleagues to name the biggest seller of CE systems. Invariably one of the two names given above would be quoted and I'd delight in telling them they were very wrong.

The biggest companies were those manufacturing DNA sequencers. Whereas proteins had proved a considerable challenge to separate in a capillary format DNA had not. The initial concept had been to use the slab gel approach and form acrylamide type gels within the capillary but this proved both difficult and unreliable. The much simpler method of using a viscous polymer in the electrolyte was remarkably effective in resolving DNA fragments. This approach in combination with sequencing chemistries using fluorescent dye terminators, laser induced fluorescent detection and multiple capillaries running in parallel CE was to be found in the majority of high throughput DNA sequencers such as the ABI 3700. These systems require speed and all that is needed qualitative results. It could be argued that without CE we would still be sequencing the human genome rather than having read the first version 10 years ago. The role of CE in the human genome project has been covered by Zhang and Dovichi . Today the need is for even faster sequencing in areas such as human medical research and forensic analysis. The original 16 capillary systems have been replaced with 96 capillary systems holding stacks of 96 well plates and 384 capillary systems are becoming available from a number of companies (Figure 5). The recently introduced megaBACE 4000 from GE Healthcare uses 384 parallel capillaries and can generates over 2.8 million bases per day. The latest development focus on removing the fluorescence detection and methods based on pyrosequencing using enzymes with chemiluminescence detection are being developed. Such CE based systems are currently being incorporated into small and inexpensive sequencers

Protein separations by CE have proved difficult due to the binding of many proteins to the silica wall of the capillary. The possible to exception to this broad statement is the ready separation of human serum/plasma proteins. The ten or so common proteins in plasma are readily separated by CE using simple borate buffers with detection at ca. 210 nm (Figure 6). The separation is rapid and gives direct quantitation unlike the tradition agarose gel methods, which are slow and require staining and densitometric measurements. Even standard analytical systems are capable of running 10 plasmas per hour. A number of years ago Beckman developed at 8 capillary clinical protein

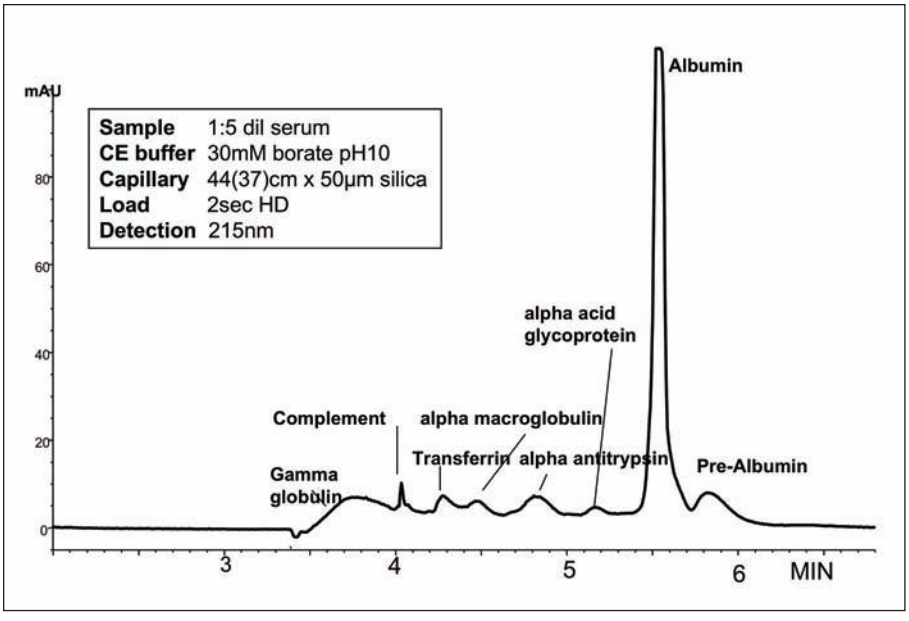


*Figure 6. CZE separation of the proteins in normal human serum.*

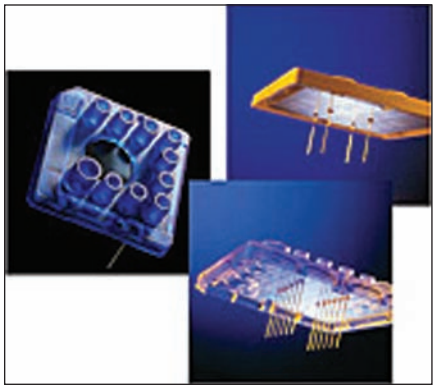

*Figure 7. Caliper chips*

analyser called the Paragon but Sebia have come to dominate this clinical market place. Their Capillaries-2 multi capillary system is designed to fit into the clinical laboratory's work flow offering pre-made buffer kits etc, is simple to operate and comes with considerable interpretation software. In addition other kits for urinary proteins, haemoglobins and immuno-subtraction are available.

Although multiple capillary CE is commonplace in high throughput genomics very few manufacturers developed such systems for the chemical and pharmaceutical analysis market probably considering the market to be too limited. An Imperial College (London, UK) spin-out company (DeltaDot (London, UK)) entered the CE market place a few years ago employing a novel detection algorithm with a conventional design of CE system. Agilent and Beckman continue to operate in the CE field and seeing the increased use of CE by biotechnology companies they both replaced their long standing instruments with new improved models last year. These systems have improved electronics, a small foot print and better detection modes with enhanced sensitivity.

### Lab on a chip.

Miniaturised separations on chip-type devices date back to 1975 when a GC on a chip was constructed at Stanford University. Electrophoresis along with electrokinetic injection revolutionised on-chip analytical devices. Micro-machined electrophoresis probably originated in the very innovative analytical group led by Michael Widmer working in the Swiss pharmaceutical company, Ciba Giegy. In 1992 they described planar glass chip technology for miniaturization and integration of separation techniques into monitoring systems - Capillary electrophoresis on a chip . Over the next decade many other groups both in academia and in various small companies developed the lab-on-a-chip concept. Devices that incorporated electrolyte loading, voltage control, sample introduction, separation and detection on a single chip were patented and then described with increasing frequency. Initially the chips used acid etched channels on glass slides but chips using more sophisticated soft lithographic techniques able to create increasing complex channels soon became commonplace. Detection usually used laser induced fluorescence since it was difficult to focus other light sources onto the narrow separation channels. Electrochemical detectors have also been employed. Plastic materials e.g. PMMA, polycarbonate, were soon being investigated to fabricate the chips. These chips were easier and cheaper to fabricate. Recently flexible films such as polyester and even paper have been investigated in potentially cheap disposable

*Figure 8. Agilent bioanalyser 2100*

devices for say point-of-care (POC) testing in GP surgeries. However most of these chip devices were characterised by a very small chip surrounded by large power supplies and laser beams usually has large or even larger than a standard CE system. The dream of a commercial complete POC system the size of a laptop still appears some way off and the ultimate goal of a mobile phone sized device is even further away. Nevertheless commercial electrophoresis on a chip systems have been around for some time., The Agilent Bioanalyzer when launched in 1999 was the first commercially available instrument to use chip technology for the analysis of DNA and RNA as well as being able to perform SDS-PAGE electrophoresis in a bench-top system. Other companies have entered this market with for example Caliper Life Sciences (Runcorn,UK) offering probably the largest range of instruments including as well as DNA, RNA and protein sizing systems, automated enzyme assays (Kinases) for drug discovery. The current status of electrophoresis on a chip has been reviewed by Arora et al.[25]

### Conclusion

Since many molecules carry an intrinsic charge electrophoretic methods will continue to be very important especially in the area of biological analyses. CE is at present undergoing something of a revival in the biotechnology field for protein analysis and to some extent for the chiral analysis of small pharmaceuticals. More systems based on lab-on-chip principles will be developed especially with respect to point of care testing in hospitals and doctors surgeries. In other areas there are hidden applications of electrophoresis e.g. immobility mass spectrometry was developed decades ago as electrophoresis in the gas phase. Yet for many biological laboratories slab gels being simple, cheap and flexible will continue to be a work-horse method for years to come.

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