Akademie věd České republiky

Teze disertace
k získání vědeckého titulu "doktor věd"
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Interfacing microchannel separations with electrospray mass spectrometry

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Ústav analytické chemie, AV ČR, v.v.i.,
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Komise pro obhajoby doktorských disertací v oboru Analytická chemie

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Table of Contents

Summary

This dissertation describes development and application of interfaces for coupling of microchannel separations with electrospray mass spectrometry. The text, based on 20 selected publications, covers mainly electrophoretic separations coupled to electrospray mass spectrometry for both capillary arrangements and chip based devices. The papers document my research activities in electrospray mass spectrometry interfacing over the past 24 years and were selected from over 100 other studies published during the 1993 – 2016. The presented work is highly experimental and since each arrangement has its own specifics, I have divided the papers into two separate sections (although both parts of the work have overlapped in time). The first part deals with capillary electrophoresis-mass spectrometry and includes eleven research papers covering various aspects of interfacing of capillary electrophoresis, performed in standard fused silica capillaries, with electrospray mass spectrometry. The second part documents the evolution of different approaches and instrumentation for on-line coupling of microfabricated devices with electrospray mass spectrometry.

Introduction

In the late 1980 – early 1990 the mass spectrometry witnessed a paradigm change when John Fenn published a series of papers describing electrospray ionization[1]. Suddenly, the analysis of large molecules, including biopolymers, could be easily achieved without fragmentation. Moreover, the multiply charged ions of peptides and proteins could be analyzed on mass spectrometers with limited mass/charge range. Within few years the mass spectrometry, once dominating only in laboratories dealing with inorganic and/or small organic molecules analyses, became an important tool for bioanalysis.

At about the same time another revolution was growing in molecular biology related to the invention of new tools for DNA analysis. In 1990 the Human Genome Project officially started[2] and after its completion little over a decade later, the outcomes are changing the way biology and medicine is practiced today. Also in the same time, capillary electrophoresis has emerged as an interesting separation tool for rapid and highly efficient analysis of ionogenic compounds[3]. In that period I was finishing my CSc. (PhD in today's world) with the focus on theory, instrumentation and applications of capillary electrophoresis[4] under the direction of Prof. Petr Boček. When the first papers, indicating the potential of CE for rapid separations of DNA fragments, have just been published[5-6] we have also started to look into the technology [7]and in 1991 I was lucky to get a postdoctoral position at the Northeastern University in Boston. Originally, my intention was to work on the research related to the Human Genome Project[8-10] with Prof. Barry L. Karger at the Barnett Institute; however, during the first year the laboratory received a brand new TSQ700 triple quadrupole electrospray mass spectrometer from Finnigan and I was asked to interface it with capillary electrophoresis. That was the start of my diversion towards the mass spectrometry coupling territory, which extended for another 10 years of my stay in Boston and continues as one of the main research activities even today in Brno. The following text will briefly review the development of both capillary electrophoresis-electrospray mass spectrometry (CE-ESI/MS) and coupling of microfluidic devices with ESI/MS. The text is largely based on review papers, which I have co-authored, but are not included in the papers selected for the presented thesis[11-14].

1. Mass spectrometry interfacing

The advances in the mass spectrometric instrumentation provide tools for high resolution and high throughput analyses of broad range of biological analytes. Given the resolving power of the new time of flight (TOF), the Fourier transform mass spectrometers (FT-MS) and Orbitrap instruments one might question the need for sample separation prior to the MS analysis. Indeed, instruments with the resolution of several hundreds of thousands have clearly demonstrated the capability for analyses of crude mixtures of hundreds of proteins and protein digests[15-16]. Although impressive, the resolution of the mass spectrometer itself does not guarantee a successful analysis. Besides the need to separate isobaric species, isomers and isoforms, the ionization suppression effects are the main limiting factors for practical use of direct MS analysis. Both main interfacing techniques used in biological mass

spectrometry, the electrospray (ESI) and matrix assisted laser desorption/ionization (MALDI), suffer from these problems. It is frequently observed, that the detected MS signals of individual species in a mixture do not correspond to their respective concentrations. In some cases the presence of a certain (often major) sample component completely suppresses the signal of other components. Although, the ionization processes are still not fully understood, it is known that signal suppression can be eliminated by separating the interfering compounds, e.g., by chromatography or electrophoresis. An example of the observed signal suppression is shown in Fig. 1.
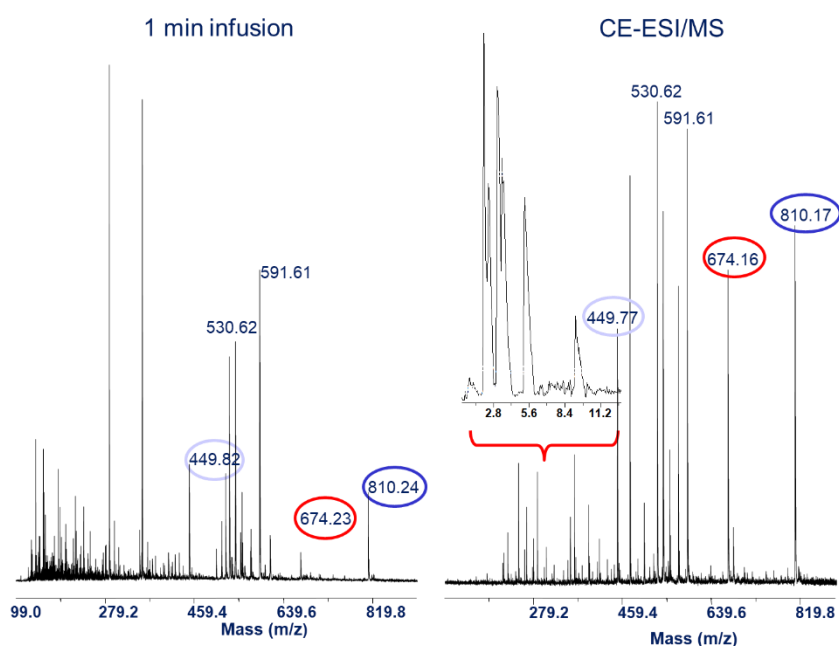

Fig. 1 Demonstration of the signal suppression in ESI/MS analysis. The left panel shows an ESI/MS spectrum averaged during a 1 min infusion of a peptide mixture. The right side shows the ESI/MS spectrum of the same sample injected into a CE capillary in 10x smaller volume and separated by CE-MS. The mass spectra behind the total ion current CE-MS trace shown in the inset were summed up to provide the corresponding total sample mass spectrum. The circled numbers were selected to point out some of the clear differences in the signal intensity. F. Foret, unpublished results.

This example is typical for practically all complex samples where the minor sample components can be completely obscured by compounds with higher concentration and/or higher ionization efficiency, e.g., higher proton affinity in the gas phase. Thus coupling of liquid-phase separation techniques with mass spectrometry (MS) is the main way for obtaining identity and structural information in many fields of bioanalysis including proteomics. Although dominated by the maturing LC/MS technology there are also other techniques playing important roles in specific bioanalytical areas. Capillary electrophoresis (CE) offers different selectivity, higher efficiency and often also shorter analysis time compared to HPLC. In addition, working with narrow, open separation capillaries and very small (nL) injection volumes may be an advantage

when the sample amount is limited or for a second dimension in multidimensional separations. Finally, once optimized, electrophoretic separation protocols can be easily scaled either for obtaining higher injected amount in longer capillaries or for speed in shorter columns or microfluidic chips.

The above mentioned arguments have been documented in a number of original and review articles dealing with CE-MS. Review articles focused on the instrumental[17-18] and wet chemistry[19] aspects as well as CE-MS application in various fields such as proteomics[20-24], glycomics[25], metabolomics[26], biomarker discovery[27-30], amino acid analysis acids[31] and/or chiral CE-MS[32]. Other CE-MS reviews specialized on the use of capillary coatings in CE-MS[33], CE-TOF/MS[34], or on-line coupling of electrokinetic chromatography and mass spectrometry[35]. Selected instrumentation as well as CE-ESI/MS applications are discussed below.

2. Instrumentation for CE-ESI/MS

A variety of ionization methods such as atmospheric pressure chemical ionization (APCI), atmospheric pressure photoionization (APPI), sonic spray ionization (SSI), thermospray ionization (TSI), matrix-assisted laser desorption/ionization (MALDI) or continuous-flow fast atom bombardment (CF-FAB) have been attempted for CE-MS coupling[36]; however, electrospray (ESI) is by far the most popular ionization technique. In principle, a CE-MS interface should accomplish four important features: (i) electrical connection for adjusting ESI potential, (ii) electrical connection to close the electrophoresis separation current, (iii) suitable outlet for direct spray of separated analytes, and (iv), in some cases, introduction of a spray liquid and nebulizing gas. For reproducible separation and stable ESI, the optimal CE/ESI/MS interface device should effectively decouple the CE and MS processes so that each could work under optimal conditions without negatively affecting the other. Physical robustness, ease of use with maximal stability and sensitivity for analyte detection as well as maintaining CE separation efficiency represent practical key characteristics required for CE-MS interfaces. A number of different interfaces to hyphenate CE and ESI/MS have been described which can be classified into the three main categories: (i) sheathless, (ii) sheath liquid (flow), and (iii) liquid junction interfaces – Fig.2. The specific class is represented by microfabricated CE devices (microchips) with integrated spray emitters. The detail discussion of most of the microfabricated (microchip) CE-MS interfacing is summarized in review articles[14,37-39] and in the section 3.
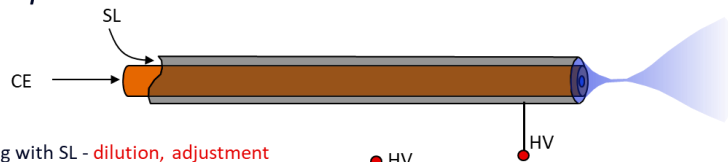
Sheathless

Very low flow (nL/min), no SL, electrolyte compatibility

HV

Coaxial sheath liquid

SL

CE
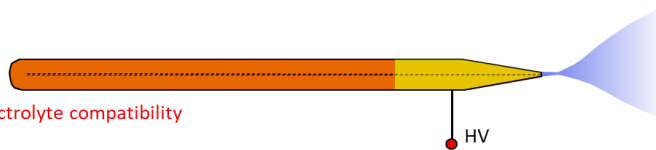
High flow (μL/min), mixing with SL - dilution, adjustment

HV

HV

Liquid junction

HV

Low flow (<100 nL/min), dilution comparable to sheathless, little adjustment
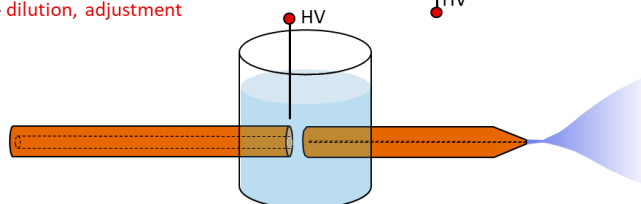Wide range of CE capillary diameters, coated capillary – no flow

Fig. 2 Scheme of the sheathless (top), sheath liquid (middle) and liquid junction interfaces

2.1 Sheathless interfaces

The sheathless interfacing for CE-MS coupling has been pioneered by the group of Smith in the late 1980s. In the first reported sheathless interface design, a fused silica capillary was terminated in a stainless steel capillary[40], and later a metalized fused silica capillary was directly used as an ESI emitter[41]. Since in the sheathless interface the fused silica capillary serves as both the separation capillary and the electrospray emitter, several methods for creating electrical contact at the ESI end of the CE capillary have been developed. The most common approaches include metal coating of the tip, inserting an electrode inside the capillary outlet, use of porous etched capillary walls or the use of a microdialysis junction[42]. Sheathless interfaces do not suffer from the dilution effect by the sheath liquid; however, as there is only one background electrolyte for separation and ionization, the separation/ionization conditions must be optimized accordingly to fulfill both needs. Reduction of the flow compared to the sheath liquid based interfacing significantly improves ionization efficiencies as well as reduces ion suppression of co-migrating analytes.

Based on the porous membrane design[43], a new front-end separation and ionization technology called CESI 8000 module with the OptiMS sprayer has recently been developed in the laboratories of Beckman Coulter. In this sheathless interface, the 3-4 cm of the distant end of the separation capillary is etched with hydrofluoric acid. The etching procedure creates a capillary with an outer diameter (OD) at the etched portion of 40 μm with a ~5 μm thick porous wall[44]. Since the porous tip is not internally tapered, the sprayer presents a good ability to spray at low flow rates while reducing the potential for clogging. The porous tip is located within a housing comprising of a

stainless steel needle, which protects the tip from physical damage. The electrical contact for the CE is achieved through the ESI needle, which is filled with a conductive liquid and for the ESI by the porous capillary protruding from the needle. The developed OptiMS sprayer technology is compatible with several mass spectrometers, plugging directly to the nanospray sources or by using specific adapters.

The porous tip interface is compatible with a very wide range of electrophoretic conditions (electrophoretic mobilities ranging from 2.9 to 233×10^{-9} $m^2 V^{-1} s^{-1}$, which covers most of the conditions generally encountered in CE[45]. The direct comparison with a sheath liquid interface clearly proved the benefits of the sheathless interfaces including generation of a very stable and robust ESI at flow rates in the nL/min range, reduced ion suppression and improved sensitivity[46]. Additional advancements in the design of the sprayer may further improve the performance in the future. For example, a number of reports dealing with multisprayers formed by frits, membranes and/or channel arrays, has been covered by a review article[47].

2.2 Sheath liquid interfaces

Coaxial sheath liquid interfaces, based on the triple tube, design developed by Smith et al.[48], are currently used on most of the commercially available instruments. The CE separation capillary itself is the center tube of the sprayer and it is surrounded by two metal tubes. The inner steel tube delivers the sheath liquid and the outer one delivers the nebulizing gas assisting in the spray stability. The sheath liquid completes an electrical circuit of the CE system and generates the necessary flow for a stable electrospray. Most of the interfaces utilize a stainless steel spray needle; however, stainless steel can oxidize and generate metal ions interfering with the analysis. The electrolysis behavior of the metal electrospray needles has been known for over 20 years[49] and the electrochemical reactions and ionization processes occurring during the ESI ionization has recently been reviewed[50]. These processes can cause clogging of the separation capillary as well as form complexes with analyzed anions resulting in decreased detection sensitivity. Therefore, other material such as platinum was found as a more suitable, especially for analysis of negative ions[51].

The sheath liquid interface allows independent optimization of the sheath liquid and the background electrolyte (BGE) compositions; however, a significant dilution of the sample occurs at the interface needle since the CE electrolyte flow rate is usually significantly lower (nL/min) than the sheath liquid (μL/min). Furthermore, a number of parameters such as CE capillary protrusion from the sprayer needle, positioning of the interface with respect to the MS orifice, applying adequate voltages for electrospray, sheath liquid composition as well as flow rates of the sheath liquid and the nebulizing gas is required to be optimized to create a stable electrospray and maintain separation efficiency and detection sensitivity[52]. Another drawback of many of the sheath liquid CE-MS instruments is the requirement to use relatively long separation capillary due to the instrument configuration (40 cm or more, typically with the inner diameter (ID) of 50 or 75 μm), leading to long separation times. Unfortunately, the requirement to use long separation capillaries is dictated by the design of the commercial instruments where the separation capillary has to reach out from the CE instrument to be

connected to the external ESI interface. Thus, while the UV absorbance can be monitored just few centimeters past the injection, the distance to the ESI interface is almost an order of magnitude greater. This contrasts with the potential for CE separation on the timescale of seconds demonstrated with capillaries narrower than 50 μm[53]. In the recent nanospray sheath-flow interface design a stable spray is achieved with very low sheath flow rates and without a pump or nebulizing gas[54]. Here, the separation capillary is placed inside a tapered glass emitter (2-10 μm id) and ESI voltage is applied via a platinum electrode placed in the sheath liquid reservoir. The capillary, the electrospray emitter, and the sheath-liquid tubing are connected via a PEEK cross. Sheath liquid is driven by electroosmosis produced by the zeta potential at the emitter surface. The sheath liquid flows over the end of the separation capillary, closing the circuit and mixing with the capillary effluent inside the tip. This design, although described as sheath liquid arrangement by the authors, may be also classified as the liquid junction arrangement.

In another, but similar design, the separation capillary was secured in a microtee assembly serving as the body of another nebulizer-free sheath liquid interface described by Lapainis et al.[55]. The PEEK tee incorporated a stainless steel metal tubing opposing the port in which the CE capillary was secured, serving as the sheath liquid tube and ESI needle. The design of these scale-down interfaces allows achieving a stable electrospray at significantly lower flow rates (250 nL/min - 2 μL/min) than commercially available sheath liquid interfaces typically operating at 4-10 μL/min flow of the sheath liquid. Although in some cases one can lower the flow in the sheath liquid interface down to a μL/min this may require a careful positioning of the separation capillary at the exit of the sheath liquid tube. Even in such a case the stability of the sheath liquid electrospray process is compromised. This can be attributed to the larger size (diameter) of the electrospray tip resulting in lower and less homogeneous electric field. The sheath liquid electrospray has to be positioned at a greater distance from the mass spectrometer sampling orifice leading to larger ESI plume size. Since the sampling orifice of current mass spectrometers is limited by the pumping speed of the vacuum system only a very small part of the ESI plume can enter the mass analyzer. Using of the nanospray emitter not only reduces the liquid flow and the size of the electrospray tip, but also allows easier spatial optimization of its position in front of the mass spectrometer resulting in enhanced ion transfer efficiency and detection sensitivity. Similar low sheath flow interfaces were described with a removable ESI sprayer for capillary and chip-based CE-MS applications[56-58].

2.3 Liquid junction interfaces

Since the first paper dealing with a liquid junction interface[59], a plethora of designs has been developed. The bodies of the liquid junction interfaces are mainly made from glass[60-61] or plastic materials such as polypropylene[62-63], polycarbonate[64] and polysulfone[65]. The use of inert materials such as glass or polysulfone minimizes the ESI chemical noise caused by plastic softeners or material degradation. In the liquid junction interface, the separation and electrospray capillaries aligned axially are separated by a small gap (20-200 μm) permanently filled with a spray liquid[66]. A fused

silica capillary with the ID of 10–50 μm with a sharpened and polished tip is usually used as the ESI emitter. The spray liquid reservoir is equipped with the electrode for connection of the ESI potential. This arrangement provides independent optimization of the CE separation and ESI conditions.

A pressurized version[67] of the liquid junction interface is capable to work with a nanospray needle (10 μm id) at flow rates of tens of nL/min[60-61,65]. To avoid a pressure driven flow in the separation capillary, both the background electrolyte reservoir and the liquid junction ESI solution reservoir are maintained at the same pressure. The detail numerical analysis describing the mass transport of analytes through the liquid junction interface has recently been presented[68]. It has been shown, that the most important parameters of the liquid junction interface effecting the transfer of analyte zones between the separation and the spray capillaries are (i) the electric field strength that controls the migration of analytes in the CE capillary and in the gap, and (ii) the pressure exerted on the gap that controls the liquid flow rate through the spray capillary. On the other hand the interface geometry, i.e., the gap width between the separation and spray capillaries can be varied in relatively broad range (20-200 μm) without a detrimental effect on the separation.

A recent design of the liquid junction interface called a junction at the tip interface consisting of the separation capillary (365 μm od) inserted as far as possible into the stainless steel hollow ESI emitter with a beveled tip has been developed by Chen and coworkers[69-71]. A space enclosed by the CE capillary exit and the inner surface of the stainless steel tip forms a flow-through microvial acting as the outlet vial and the terminal electrode. The junction is filled with a spray liquid, supplied from the reservoir at a flow rate of ~100 nL/min, supporting a stable electrospray with minimized sample zone dilution. Numerical simulation describing the mass transport of the analyte through the junction at the tip interface was verified by CE-MS experiments[72], proving the laminar flow profile in the microvial with no broadening of the analyte zone (peak shape) by the spray liquid.

3. Microfluidics

In the early 1990 the Human Genome Project was still searching for technologies suitable for analyzing the (then) immense amounts of Sanger sequencing products. Many bet on the new developments in mass spectrometry and while impressive results were obtained early on especially with MALDI-TOF instruments[73], the obstacles related to the total time of analysis, data processing, instrument size and cost finally led to the development of different technologies. One technology many groups moved into was microfabrication using photolithography developed originally for electronics. In fact it was already tested for microfabrication of a gas chromatograph as early as in 1979[74], as shown in Fig. 3.
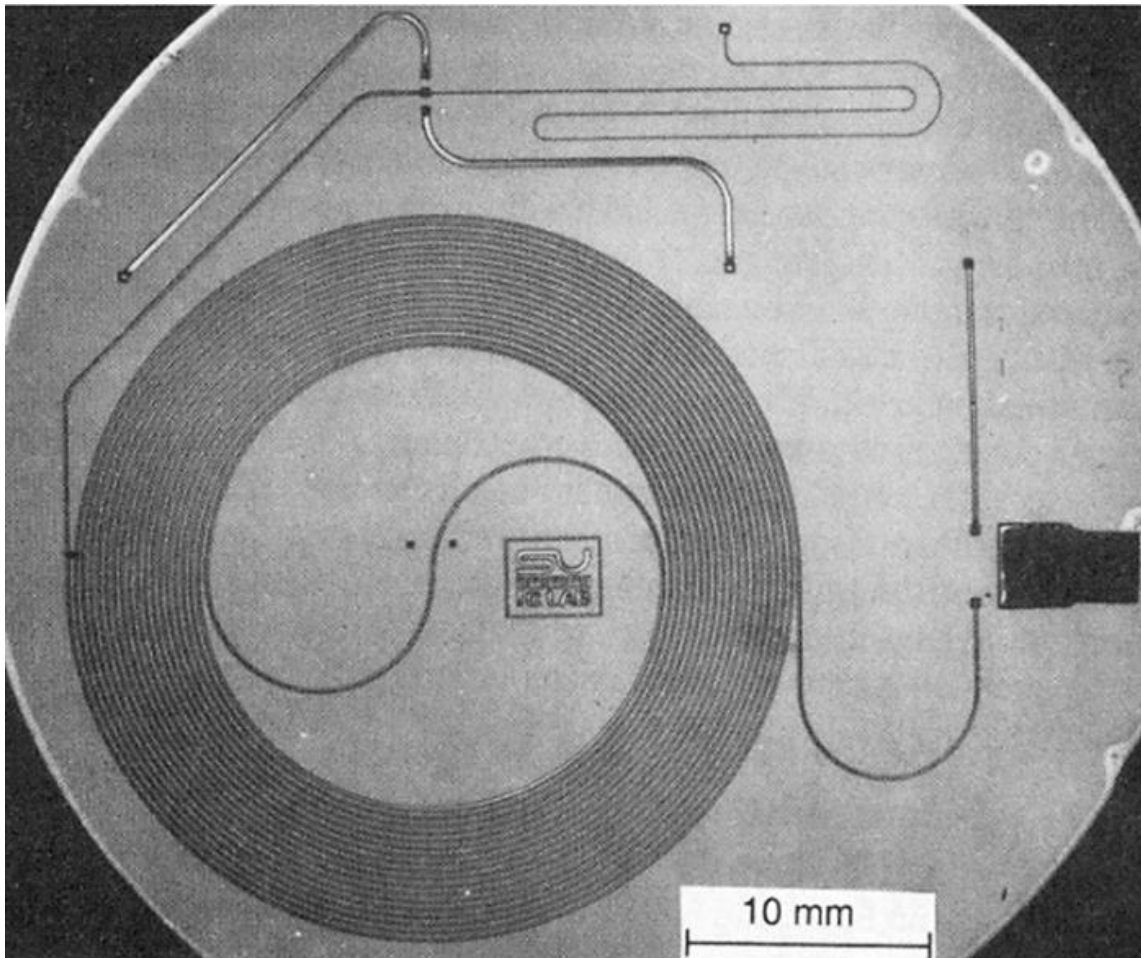
Fig. 3 A Gas Chromatographic Air Analyzer Fabricated on a Silicon Wafer[74].

The main advantage of miniaturization was in the possibility of creating highly parallel systems with a small footprint and high speed of operation. Indeed, the results of this research have materialized in the next generation of DNA sequencers being used today. In mid 1990s several groups published exciting papers on microfabrication of parallel channel capillary electrophoresis systems for high throughput DNA sequencing. One example of such a system as described by Mathies et al.[75] is in Fig.4.
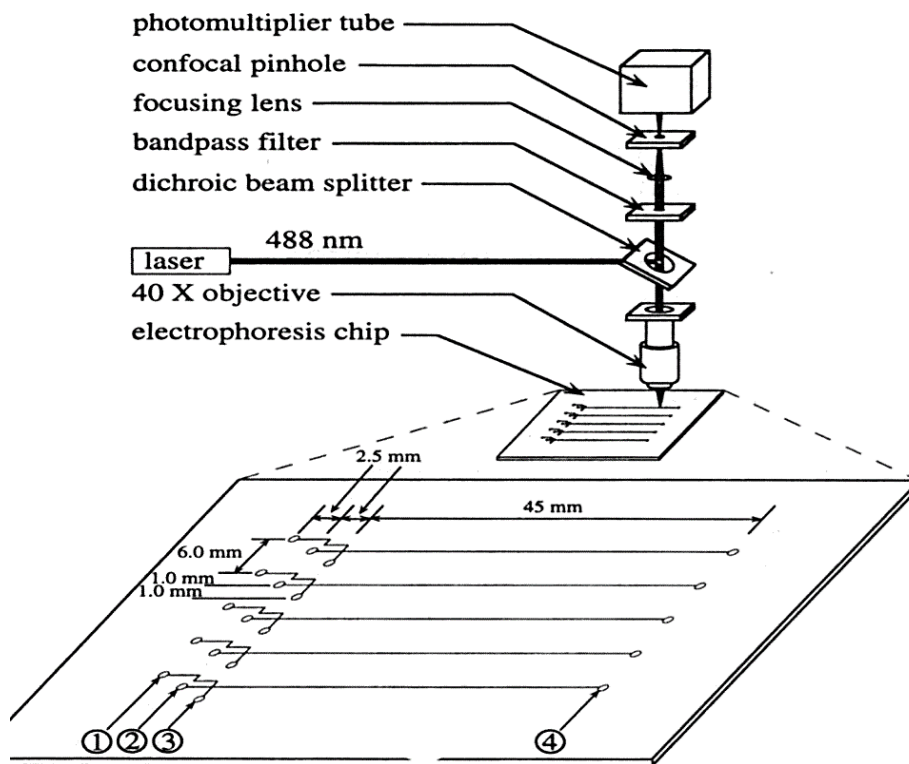
Fig. 4 Ultra-high-speed DNA fragment separations using microfabricated capillary array electrophoresis chips[75].

At that time we were developing a capillary array DNA sequencing technology and felt that we might be missing out on the microfabrication. As a group Leader at the Barnett Institute, I was under increasing stress to move into microfluidics. Since we had neither experience nor the equipment, I have resisted for some time. Eventually, I realized that microfluidics could be potentially useful for the electrospray mass spectrometry and filed an invention disclosure. Soon we have filled a full patent application[76] with colleagues from the electrical engineering microfabrication lab and a new postdoc was hired to work on the project. What seems quite logical today was quite difficult to start then and we had to improvise, especially in the early stages of the work. It is interesting to note that the Human Genome Project was eventually finished with the standard capillary array technology at both the Celera Genomics (private part of the DNA sequencing) and Molecular Dynamics (the government funded part); however, the research on microfluidic sequencers generated a basis of a new area of analytical instrumentation, including mass spectrometry coupling. The basic arrangements of the devices we were testing at that time as disclosed in the patent are in Fig. 5.

Since microfabrication of pointed electrospray tips in glass was difficult, our first devices used a simple channel opening on the polished surface (sometime silanized) of the glass chip edge. While such an arrangement was not suitable for coupling with separations, infusion experiments worked quite well. Soon it turned out that the timing of the work was right since all major instrument manufacturers licensed the technology and eventually brought commercial instruments to the market.
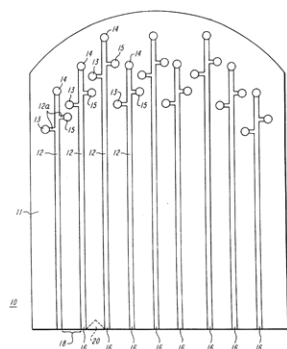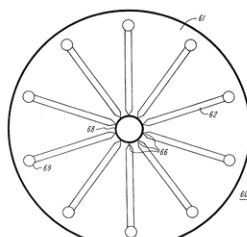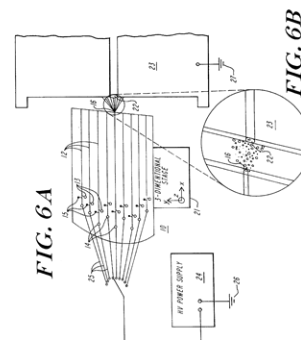
FIG. 1A

FIG. 3

FIG. 6A   FIG. 6B

Fig. 5 Microflidic designs for coupling with ESI/MS[76].

3.1. Microfluidics interfacing with ESI-MS

There are three important issues that must be addressed in the design of a microchip-MS interface. First, an approach must be developed to ensure high electrospray ionization efficiency from the microfabricated device, in order to obtain high sensitivitiy. Second, if separations are to be performed on the chip, the contribution of the interface to band broadening must be minimized. Third, since most of current applications use electrical forces to control fluid flows on the chip and since MS detection occurs off-chip, an effective approach must be found to direct the fluids towards the MS interface.

Spray generation from the microchip flat surface.

In early reports where microdevices were used for infusion ESI-MS analysis, electrospray was initiated directly from the channel opening on the flat surface of the chip[77-79]. The open channel electrospray properties were studied in more detail for devices made of a dielectric, non-wetting material[80]. In accord with previous experimental results[81], it was concluded that the electrospray activated from a small opening on a flat hydrophobic surface can have performance close to that of a needle arrangement[82]. Although the ability to generate electrospray directly from the chip surface was clearly demonstrated, the flat edge may not be suitable for direct coupling with on-chip separations. Close inspection of the electrospray cone revealed a volume of tens of nL – Fig. 6. Thus,for microchip separations where peak volumes are typically below ~5 nL, any separation would be lost in the dead volume of the electrospray cone. As with column separations, a sharp electrospray tip is required in order to minimize dead volumes and to improve ionization efficiency.
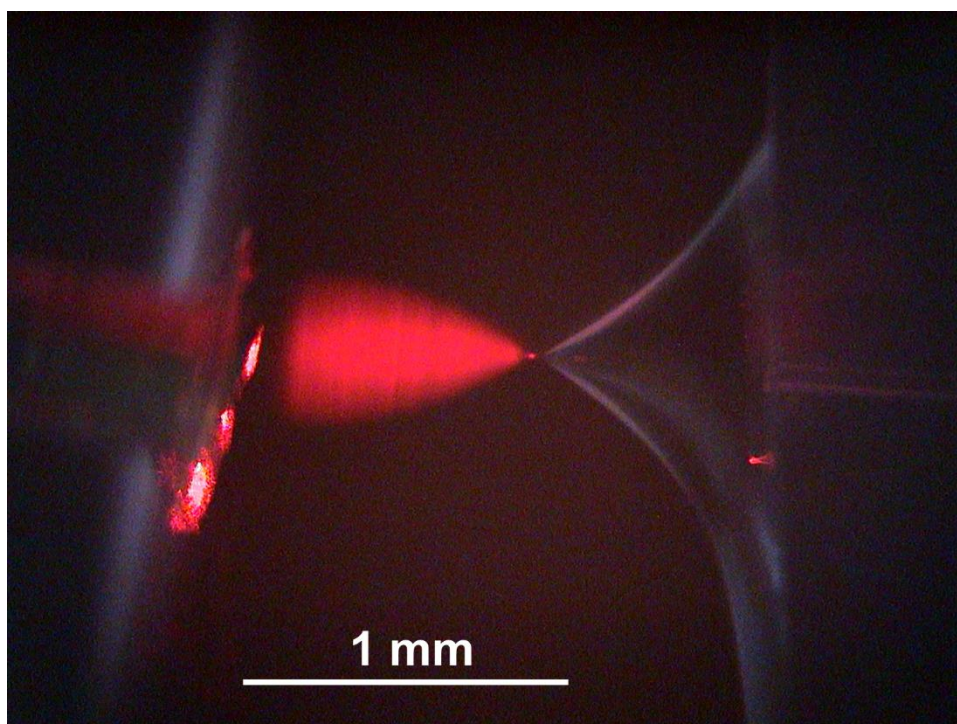
Fig. 6 Image of the electrospray plume (illuminated by a red laser) generated off the microchannel ending on the edge of the glass chip. The droplet formed at the channel exit is clearly visible. F. Foret – unpublished.

Spray generation from capillary emitters inserted in the microchip.

A variety of approaches have been taken to generate electrospray by inserting capillary emitters in the microchip device, resulting generally in performances comparable to those found for microcolumn separations. Either an electrospray tip or a fused silica capillary transfer line was inserted in the microchip body[83-84] or a liquid junction configurations with a removable electrospray tip have been developed[85-86]. Some of the arrangements developed during my work form part of this thesis – papers 14-17.

Microfabricated electrospray emitters.

Batch-generation of microchips with integrated electrospray emitters/tips can result in improved emitter reproducibility, and the potential for simple, disposable devices. However, the microfabrication of fine electrospray tips as an integral part of a microdevice is not a trivial task, and suitable microfabrication procedures are still under development. Robust, hollow needle structures (electrospray emitters with tapered tips having 5 x 10 μm rectangular openings, that extended 1 mm beyond the edge of the substrate), were fabricated from parylene polymer layers deposited on a silicon substrate[87] and microfabricated electrospray nozzles with high aspect ratio (10 μm ID x 50 μm depth) were constructed on the planar surface of a silicon substrate using deep reactive ion etching[88-89]. These silicon ESI Chips™ in a 100 nozzle format are presently commercialized - www.advion.com. Alternative techniques have used a combination of low pressure chemical vapor deposition, pattern transfer, reactive ion

etching and sacrificial layer etching for the fabrication of miniaturized polysilicon-based ESI emitters[90].

The microfabrication of disposable plastic microdevices is attractive from the commercial perspective and a number of fabrication procedures can be found in the literature. For electrospray generation, the parylene film was micromachined in a triangular shape by lithography and etching[91]. In another approach, the microfluidic system and the electrospray exit nozzle were fabricated by plasma etching in polyimide[92]. In addition, electrospray emitters were fabricated from SU-8 epoxy resin by photolithography[93], from polyethylene terephthalate[94] and polycarbonate substrates[95] by laser ablation, from poly(dimethylsiloxane) by casting[82], from poly(methylmethacrylate) by injection molding [96-97], micromilling[98] and/or mechanical cutting[99]. Plastic systems are also attractive for low volume multiple channels systems as documented for glutamate release from neuronal cells[100] or small-volume proteomics[101].

It is interesting to note that glass, the most common material in microfluidics, has only recently been rediscovered as a material for integrated ESI devices. For laboratory use the group of Detlev Belder uses a mechanical cutting of the glass around the channel exit followed by flame pulling. While this approach may not be suitable for mass production it brings a good potential for high quality "proof-of-principle" research, including MS studies of rapid chemical synthesis[102] and or chip based HPLC[103]. A more streamlined way of fabrication of glass microdevices with integrated was recently developed by R. Kostiainen et al.[104 -105]. The group of M. Ramsey has been probably the most active group in the development of CE-ESI/MS microdevices, which are now commercially available via the 908devices company – www.908devices.com. Their works cover a wide area of systems for both electrophoresis[106-108] and two dimensional separations combining the CE with chromatography[109].

4. Concluding remarks

Electrospray/MS coupling is one of the most important tools in the current (bio)analytical instrumentation. Mature CE equipment has been on the market for quite a while and new generation of instruments is under development. It is worth noting that at the peak of the "irrational exuberance" of the stock market around the year 2000 microfabrication and microfluidics were the banners for success. Many startup companies made their fortunes overnight when going public at that time. Several years later only a few of the "old timers" remained in the business; however, new startups are still being formed. In his editorial "Microfluidics, the Ultrahigh-Throughput Underachiever" in the GenomeWeb News (5/14/03) the senior editor John S. MacNeil calls this period as a time of" frantic search for the one application that will force reluctant customers in academia, biotech, and big pharma to go whole hog on microfluidics, since the whole concept might just be too powerful not to succeed eventually."

As the success of the number of next generation sequencers documents, the new technologies are and will continue changing many fields in the science, technology and medicine. Take the applications of microfluidics in chemical analysis as an

example. Although the first examples of the development of the microfabricated instrumentation can be traced back to the mid-seventies, without any doubt the major trend of miniaturization and integration of analytical processes and instrumentation has been witnessed only in the past ten years, or so. The figure shown below clearly demonstrates the trend – Fig.6.
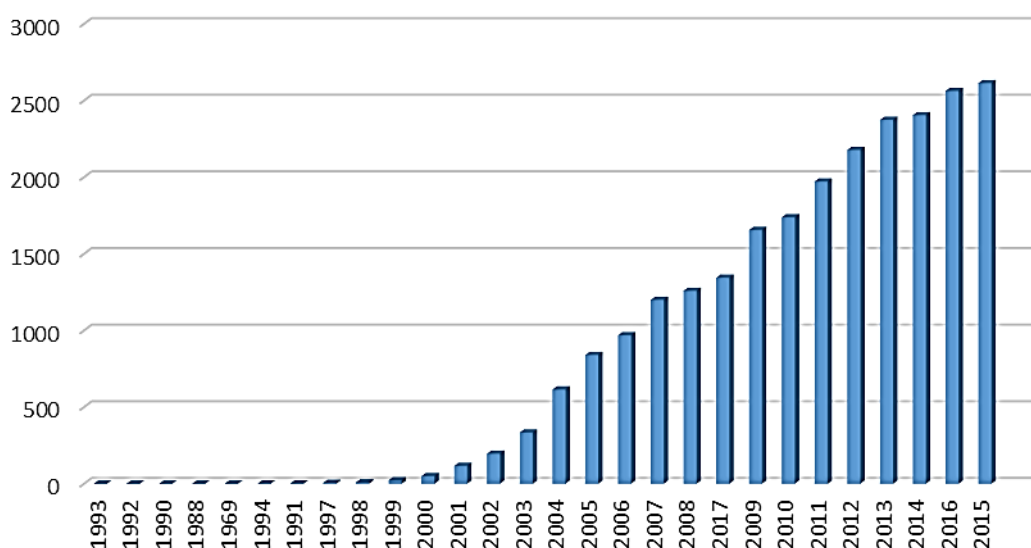

Fig. 6. Plotting the occurrence of the word „microfluidic"reveals that the number of scientific articles listed in the PubMed database (www.ncbi.nlm.nih.gov/PubMed) started increasing exponentially in just the same time when the stock market bubble burst. Data from June 8, 2017.

It is anticipated that microfluidics[110] (Lab-on-a-chip) will play an important role in the new instrumentation for high-sensitivity/high-throughput analyses. The main advantages of the technology include speed of analysis, minimum consumption of reagents and samples, integration of functional elements and possibility to create massively parallel systems for high throughput. Another important feature of the microfabrication technology from the prospective of a separation scientist is the fact that leak-free, zero dead volume junctions can easily be produced. Additionally, the reduction of the instrument size leads to lower requirements for the laboratory space. In an increasing number of cases, mass spectrometry coupling is required and this trend will keep growing as documented by the recent successful introduction of the zip-chip CE-MS device by the 908 devices company[111]. Fabrication technologies include the processes used commonly in electronics, e.g., photolithography and wet chemical etching or reactive ion etching in glass or silicon. Structures as small as few nm can currently be fabricated in microelectronics; however, two to three orders of magnitude larger structures are more common in microfluidics. Precision injection molding can be used for replication in plastic materials. The advantages of the new technologies are clear and the potential applications are endless.

The Human Genome Project finished well ahead of schedule thanks also to the massive application of capillary electrophoresis. While the majority of today's analytical applications relate to HPLC separations, with capillary electrophoresis being

a niche use, the general belief is that CE might grow rapidly in the near future for applications in protein (top-down proteomics), glycan and larger biopolymers separations in general. With the current advances in life sciences there is a continuously increasing need for new analytical tools. Besides chromatographic techniques, capillary electrophoresis is the only high resolution separation alternative. New protocols are being developed for CE separations of proteins (e.g., antibodies), peptides and oligosaccharides, where the separation efficiency typically exceeds that of chromatography. While the sample loading capacity of CE is often mentioned as a serious drawback, it can be significantly improved by on-line preconcentration techniques. In addition, this lower sample capacity turns into an advantage when dealing with limited sample quantities. The electrospray interfacing is clearly the key component required for the successful deployment of the CE-MS in practice. While one can argue that many of the interfaces are alike, it is the technical details, designer/operator skills and a particular application, which lead to the use of a particular design. Many interface designs have been described in the past 20 years; however, a universal solution to all the needs is difficult to find. The following selected papers describe some of our contributions to the field.

5. Brief description, impact factors and numbers of citations of papers used in the dissertation

Part one – papers on capillary electrophoresis-electrospray/mass spectrometry

1.
Thompson, T. J., Foret, F., Vouros, P., Karger, B. L. Capillary Electrophoresis-Electrospray Ionization Mass Spectrometry: Improvement of Detection Limits Using On-column Transient Isotachophoretic Sample Preconcentration. Anal.Chem., 1993, 65, 900-906.
IF = 4.075;   174 CIT

This work belongs to a series of papers addressing the limited loading capacity of CE. Based on the knowledge of ITP principles we have developed a protocol for on-column sample concentration allowing injections of much larger sample volumes than common in regular CE. The term transient ITP, used in this work, later became widespread in the literature. Mixtures of model proteins have been separated in the cationic mode using a coated capillary and have been analyzed by mass spectrometry coupled on-line to an electrospray interface with a coaxial sheath flow arrangement. An interesting phenomena was observed in zones of lactoglobulins forming a non-covalent complex with the 6-aminocaproic acid present in the BGE. Compared to regular CE the detection limits could be improved by at least a factor of 100. Advantages and limitations of the technique with respect to the very narrow ITP zones were discussed.

2.
Foret, F., Thompson, T. J., Vouros, P., Karger, B. L., Gebauer, P., Bocek, P. Liquid sheath effects on the separation of proteins in capillary electrophoresis electrospray mass spectrometry. Anal. Chem., 1994, 66, 4450-4458.
IF = 4.609;   133 CIT

In previous experiments, we have noticed that different compositions of the BGE and sheath liquid can influence the migration of zones in CE-ESI/MS. In this joint study between the Barnett Institute, Boston and IACH, Brno we have described the ionic migration in CE-ESI/MS with a coaxial sheath liquid interface. Formation of moving ionic boundaries inside the separation capillary was observed. These ionic boundaries, which can lead to delays, inversions in migration order and/or loss of resolution, were studied both theoretically and experimentally. Based on the results of the modelling of the ionic migration it was shown that even difficult to-spray electrolytes (such as phosphate-containing buffers) can be used for the CE separation with properly selected background electrolyte counterions.

3.
Foret, F., Kirby, D. P., Vouros, P., Karger, B. L. Electrospray Interface for Capillary Electrophoresis-Electrospray Mass Spectrometry with Fiber-Optic UV Detection Close to the Electrospray Tip. Electrophoresis, 1996, 17, 1829-1832.
IF = 2.467;   12 CIT

This technical work takes advantage of experimenting with optical fibers and UV absorbance detection back at IACH in Brno. The early ESI/MS instruments suffered

from frequent instabilities and putting a UV detector just a couple of centimeters from the exit of the separation capillary was very useful in troubleshooting the problems since it provided precise information about the time when UV-active zones enter the electrospray and allowed easy location of analyte mass information in the ion current profile. In addition the integration of the fiber optic detector directly into the ESI interface allowed using of short separation capillaries, saving experimental time on an expensive instrument shared by several users.

4.
Foret, F., Zhou, H., Gangl, E., Karger, B. L. Subatmospheric electrospray interface for coupling of microcolumn separations with mass spectrometry. Electrophoresis, 2000, 21, 1363-1371.
IF = 3.385;   51 CIT

As the experiments with different interface concepts proceeded, it was clear that a single pointed electrospray tip with minimum diameter provides the best stability and ionization efficiency. Such ESI tips can be used in the liquid junction arrangement. A low flow rate of a spray fluid is needed for the transport of electrophoretic zones into the ESI tip. Here the flow was generated by lowering the pressure in the electrospray chamber creating a subatmospheric electrospray. The previously developed fiber optic UV detector was also incorporated into the system and a chain of optically controlled photoresistors was used to adjust the electrospray voltage without the need for an additional high voltage power supply. Since the electrospray did not depend on fluid delivery from the separation column, coated capillaries without electroosmotic flow as well as capillaries with electroosmotic flow could be used for CE and capillary LC separations with separation efficiencies reaching several hundreds of thousands theoretical plates. At the time of publication these were probably the best results in its class.

5.
Křenková, J., Bílková, Z., Foret, F. Characterization of a monolithic immobilized trypsin microreactor with on-line coupling to ESI-MS. J. Sep. Sci. 2005, 28, 1675-1684.
IF = 1.829;   57 CIT

After the return back to Brno in 2001, we have started looking for new research directions for adding functionality into the CE-MS protocols. It was the time when proteomics techniques were under rapid development and enzymatic digestion was one of the most important processes. In this work we have prepared and characterized a miniaturized trypsin flow-through capillary reactor for on-line coupling with an ESI-TOF mass spectrometer. The enzyme was covalently immobilized on poly(glycidyl methacrylate-co-ethylene dimethacrylate) monolith prepared in a 75 μm ID fused silica capillary resulting in a bioreactor with high local concentration of the proteolytic enzyme. Although one can expect that some trypsin molecules got inactivated during the immobilization (the enzyme immobilization was not oriented), at flow rates of 50–300 nL/min complete protein digestion was achieved in less than 30 s at 25° C with the sequence coverage of 80% (cytochrome c). This is comparable to a 3 h digestion in solution at 37ºC. Besides the good performance at laboratory temperature, the

bioreactor also performed well at lower pH compared to the standard in-solution protocols.

6.
Kusý, P., Klepárník, P., Aturki, Z., Fanali, S., Foret, F. Optimization of pressurized liquid junction nanoelectrospray interface between capillary electrophoresis and mass spectrometry for reliable proteomic analysis. Electrophoresis, 2007, 28, 1964-1969.
IF = 3.609;   20 CIT

During a joint project with our long-term Italian collaborators, we have designed liquid junction CE-ESI/MS interface for use at the CNR in Rome. The pressurized system was designed for easier operation with the available mass spectrometer and was optimized for analyses of proteins and peptides with the separation and spray capillaries fixed in a pressurized spray liquid reservoir equipped with the electrode for connection of the electrospray potential. During optimization, the transfer of the separated zones between the separation and electrospray capillaries was monitored by UV absorbance and contactless conductivity detectors placed at the outlet of the separation capillary and inlet of the electrospray tip, respectively. This arrangement allowed independent monitoring of the effects of pressure, CE voltage and geometry of the liquid junction on the spreading and dilution of the separated zones during passage through the interface.

7.
Krenkova, J., Kleparnik, K., Foret, F. Capillary electrophoresis mass spectrometry coupling with immobilized enzyme electrospray capillaries. J. Chromatogr. A, 2007, 1159, 110-118.
IF = 3.641;   48 CIT

Based on the experience with the monolithic immobilized enzymatic reactors we have tested the use of narrow capillaries with the enzyme immobilized on the fused silica surface. These open tubular capillary enzyme reactors were tested for rapid protein digestion and on-line integration into a CE-ESI/MS system. Narrow bore (10 μm ID) capillaries were used to minimize the diffusion time of analyte molecules towards the surface immobilized enzyme and to maximize the surface-to-volume ratio. Extremely small protein amounts (atto-femtomoles loaded) could be digested within few seconds transition time. Thus, a protein mixture was injected and after the CE separation individual separated proteins were digested by pepsin prior to entering the ESI/MS.

8.
Krenkova, J., Kleparnik, K., Grym, J., Luksch, J., Foret, F. Self-aligning subatmospheric hybrid liquid junction electrospray interface for capillary electrophoresis. Electrophoresis 2016, 37, 414–417.
IF = 2.482;   3 CIT

In an attempt to design a user friendly instrumentation we have designed a self-aligning subatmospheric hybrid liquid junction electrospray interface for CE eliminating the need for manual adjustment by guiding the separation and electrospray capillaries in a microfabricated liquid junction glass chip at a defined angle. Both the ESI and

separation capillaries were inserted into the microfabricated part until their ends touched. The resulting distance between the capillary openings was defined by the angle between capillaries. The microfabricated part contained channels for placement of the capillaries and connection of the external electrode reservoirs. It was fabricated using standard photolithographic/wet chemical etching techniques followed by thermal bonding. The liquid junction was connected to a subatmospheric electrospray chamber inducing the flow inside the ESI needle. After presenting the results at the ASMS conference in Minneapolis we were approached by Agilent Technologies with an offer of a joint research grant. The collaboration continues with significant financial and instrumental support from Agilent. Two master degree thesis were finished during the work, one German student has worked in Brno for a month and another will spend winter semester in Brno this year. It is anticipated, that this research will lead to a commercialization of the interface.

9.
Tycova, A., Foret, F. Capillary electrophoresis in an extended nanospray tip–electrospray as an electrophoretic column. J.Chromatogr. A, 2015, 1388, 274–279.
IF = 3.926;   12 CIT

The most challenging instrumental aspect in CE-MS is striking the balance between the stability and reproducibility of the signal and required sensitivity of the analysis in terms of both the concentration LOD and minimum injected amount. One of the long term goals of our work is development of tools for chemical analyses of single cells. The use of very narrow emitters is necessary to minimize dilution of the cell content. Since at constant voltage the current in CE is inversely proportional to the second power of the capillary diameter we have speculated that at certain low diameter (depending on the conductivity of the BGE) the CE current could equal the ESI current. Under such a condition the CE-MS coupling would not require any interface since the CE separation would be driven by the ESI current. In this work we have explored such an "interface-free" approach, where the CE-MS analysis was performed in narrow bore (<20 μm ID) electrospray capillaries ending in an electrospray. The performance of this simplest possible CE-MS system was tested on peptide separations from the cytochrome c tryptic digest. The subnanoliter sample consumption and sensitivity in the attomole range was achieved.

10.
Tycova, A., Prikryl, J., Foret, F. Reproducible preparation of nanospray tips for capillary electrophoresis coupled to mass spectrometry using 3D printed grinding device. Electrophoresis 2016, 37, 924-30.
IF = 3.981;   1 CIT

The outcome of the work described in the previous and following papers strongly depends on the use of high quality fused silica capillary nanospray tips. Achieving of reliable and reproducible electrospray/MS signal is critical; however, reproducible (laboratory) preparation of such tips is a challenging task. In this work, we have designed a low-cost grinding device assembled from 3D printed and commercially easily available components allowing to achieve maximum symmetricity, surface smoothness and repeatability of the conus shape. Moreover, the presented grinding

device brings the possibility to fabricate the nanospray emitters of desired dimensions and tip angle. The prepared tips were tested and compared for analyses of reserpine, rabbit plasma, and aminoacids mixture. It was shown that the best results can be obtained with the lowest tip angle (below 30°).

11. Tycova, A., Vido, M., Kovarikova, P., Foret, F. Interface-free capillary electrophoresis-mass spectrometry system with nanospray ionization—Analysis of dexrazoxane in blood plasma. J.Chromatogr. A, 2016, 1466, 173-179.
IF = 2.744; 5 CIT

The newly developed interface-free capillary electrophoresis-nanospray/mass spectrometry system (CEnESI/MS) was applied for rapid analysis of the cardioprotective drug dexrazoxane and its hydrolysed form ADR-925 in deproteinized blood plasma samples. The aim of this study was to test the simplest possible CE-nESI/MS instrumentation for analyses of real samples. This interface-free system, utilizing single piece of a narrow bore capillary as both the electrophoretic separation column and the nanospray emitter, was operated at a flow rate of 30 nL/min. Excellent electrophoretic separation and sensitive nanospray ionization was achieved with the use of only one high voltage power supply. In addition, hydrophobic external coating was developed and tested for additional stability of the nanospray ionization. To our knowledge this is the first study devoted to the analysis of dexrazoxane and ADR-925 by capillary electrophoresis-mass spectrometry.

Part two – papers on microfluidics-electrospray/mass spectrometry

12.
Xue, Q., Foret, F., Dunayevskiy, Y. M., Zavracky, P. M., McGruer, N. E., Karger, B. L. Multichannel Microchip Electrospray Mass Spectrometry. Anal.Chem., 69, 1997, 426-430.
IF = 4.743; 323 CIT

This is the first published study demonstrating direct electrospray coupling of a microfabricated glass chip with mass spectrometer (ESI-MS). The microchip device was fabricated by standard photolithographic, wet chemical etching, and thermal bonding procedures and the ESI high voltage was applied individually from each reservoir for spraying sample sequentially from each channel. With the sampling orifice of the MS grounded, it was found that a liquid flow of 100-200 nL/min was necessary to maintain a stable electrospray. The detection limit of the microchip MS experiment for myoglobin was found to be in the nanomolar range. Samples in 75% methanol were successfully analyzed with good sensitivity, as were aqueous samples.

13.
Xue, Q. F., Dunayevskiy, Y. M., Foret, F., Karger, B. L. Integrated multichannel microchip electrospray ionization mass spectrometry: Analysis of peptides from on-chip tryptic digestion of melittin. Rapid Commun. Mass Spectrometry 1997, 11, 1253-1256.
IF = 3.343; 90 CIT

In continuation of our work to develop an integrated multichannel microchip interfaced to electrospray mass spectrometry (ESI-MS), this paper demonstrates one of several applications of this approach in monitoring tryptic digestion products. The multichannel microchip allowed integration of sample preparation onto the microchip to facilitate the analysis process. Melittin was selected as a model oligopeptide because it possesses a cluster of four adjacent basic residues which enable probing the site specificity of trypsin as a function of digest times. Reactions were performed on-chip in different wells for specific time periods and then analyzed by infusion from the microchip by ESI-MS, using leucine enkephalin as internal standard. The rate of formation and disappearance of the molecular ion and individual fragments was followed for a melittin to trypsin concentration ratio of 300:1. The results indicate the potential of integrating enzymatic reactions with multichannel microchip ESI-MS for automated optimization of reaction conditions while consuming only small amounts of sample.

14.
Zhang, B., Liu, H., Karger, B. L., Foret, F. Microfabricated devices for capillary electrophoresis-electrospray mass spectrometry. Anal. Chem., 1999, 71, 3258-3264.
IF = 4.555; 200 CIT

This work described two fundamental approaches for the coupling of microfabricated devices to electrospray mass spectrometry (ESI-MS). Both approaches integrated sample inlet ports, preconcentration sample loops, the separation channel, and a port for ESI coupling. In one design, a modular, reusable microdevice was coupled to an external subatmospheric electrospray interface using a liquid junction and a fused silica transfer capillary. The transfer capillary allowed the use of an independent electrospray interface as well as fiber optic UV detection. In the second design, a miniaturized pneumatic nebulizer was fabricated as an integral part of the chip, resulting in a very simple device. The on-chip pneumatic nebulizer provided control of the flow of the electrosprayed liquid and minimized the dead volume associated with droplet formation at the electrospray exit port. Thus, the microdevice substituted for a capillary electrophoresis instrument and an electrospray interface - traditionally two independent components.

15.
Zhang, B., Foret, F., Karger, B. L. A Microdevice with integrated liquid junction for facile peptide and protein analysis by capillary electrophoresis/electrospray mass spectrometry Anal. Chem., 2000, 72, 1015-1022.
IF = 4.587; 149 CIT

This work extends the previous design into an design integrating (a) sample inlet ports, (b) the separation channel, (c) a liquid junction, and (d) a guiding channel for the insertion of the electrospray capillary, which was enclosed in a miniaturized subatmospheric electrospray chamber of an ion trap MS. The replaceable electrospray capillary was precisely aligned with the exit of the separation channel by a microfabricated guiding channel. No glue was necessary to seal the electrospray capillary. This design allowed simple and fast replacement of either the microdevice or the electrospray capillary. The performance of the device was tested for CE-MS of peptides, proteins, and protein tryptic digests. On-line tandem mass spectrometry was used for the structure identification of the protein digest products. High-efficiency/high-resolution separations could be obtained on a longer channel (11 cm on-chip)

microdevice, and fast separations (under 50 s) were achieved with a short (4.5 cm on-chip) separation channel with the separation efficiency comparable to that obtained from conventional capillary electrophoresis.

16.
Liu, H., Felten, C., Xue, Q., Zhang, B., Jedrzejewski, P., Karger, B. L., Foret, F. 2000. Development of multichannel devices with an array of electrospray tips for high-throughput mass spectrometry. Anal. Chem., 2000, 72, 3303-3310.
IF = 4.587;   93 CIT

This work, describing multichannel devices with an array of electrospray tips for high-throughput infusion electrospray ionization mass spectrometry (ESI-MS), has an interesting origin. The prototype plastic devices were fabricated by casting from a solvent-resistant resin which we were using a decade earlier at the institute in Brno. The sample wells on the device were arranged in the format of the standard 96-microtiter well plate, with each sample well connected to an independent electrospray exit port via a microchannel with imbedded electrode. A second plastic plate with distribution microchannels was employed as a cover plate and pressure distributor. Nitrogen gas was used to pressurize individual wells for transport of sample into the electrospray exit port. The device was placed on a computer-controlled translation stage for precise positioning of the electrospray exit ports in front of the mass spectrometer sampling orifice and allowed very high throughput and duty cycle, as well as elimination of any potential sample carryover. High-throughput ESI-MS was demonstrated by analyzing 96 peptide samples in 480 s, corresponding to a potential throughput of 720 samples/h. As a model application, the device was used for the MS determination of inhibition constants of several inhibitors of HIV-1 protease. The photograph of the device was presented on the cover of Analytical Chemistry.

17.
Zhang, B., Foret, F., Karger, B. L. High throughput microfabricated CE/ESI-MS: automated sampling from a microwell plate Anal. Chem., 2001, 73, 2675-2681.
IF = 4.532;   98 CIT

In this work we have developed a prototype for automated high-throughput CE-ESI/MS incorporating not only the CE separation and ESI ionization but also sample injection and channel flushing after analyses. The samples were injected directly from a standard microwell plate and a miniaturized subatmospheric electrospray interface was used for ESI ionization. The microdevice was attached to a polycarbonate manifold with external electrode reservoirs equipped for electrokinetic and pressure fluid control. A computer-activated electropneumatic distributor was used for both sample loading from the microwell plate and washing of channels after each run. Removal of the electrodes and sample reservoirs from the microdevice structure significantly simplified the chip design and eliminated the need both for drilling access holes and for sample/buffer reservoirs. The external manifold also allowed the use of relatively large reservoirs that are necessary for extended time operation of the system.

18.

Grym, J., Otevřel, M., Foret, F. Aerodynamic mass spectrometry interfacing of microdevices without electrospray tips. Lab Chip, 2006, 6, 1306-1314.
IF = 5.821; 10 CIT

In the early 2000s we have started a collaboration between the Brno group and the Gyros AB in Uppsala, Sweden on adapting the ESI to the microfluidic devices fabricated in the compact disk format. After spending some time on the fabrication of ESI tips on the edge of the plastic microdevice, we have decided to test a flat channel opening and an external adapter assisting in formation and transport of the electrosprayed plume from the multichannel polycarbonate microdevice. The compact disk sized microdevice was designed with radial channels extending to the circumference of the disk. Electrospray was initiated directly from the channel openings by applying high voltage between sample wells and the entrance of the external adapter. The formation of the spatially unstable droplet at the electrospray openings was eliminated by air suction provided by a pump connected to the external adapter. Compared with the air intake through the original mass spectrometer sampling orifice, more than an order of magnitude higher flow rate was achieved for efficient transport of the electrospray plume into the mass spectrometer. Additional experiments with electric potentials applied between the entrance sections of the external adapter and the mass spectrometer indicated that the air flow was the dominant transport mechanism. Basic properties of the system were tested using computer modeling and characterized using ESI/TOF-MS measurements of peptide and protein samples.

19.

Tomas, R., Yan, L., Krenkova, J., Foret, F. Autofocusing and ESI-MS analysis of protein digests in a miniaturized multicompartment electrolyzer. Electrophoresis 2007, 28, 2283–2290.
IF = 3.609; 11 CIT

This paper is the result of an informal collaboration with the Swiss startup Diagnoswiss in Lausanne developing polyimide based microfluidics. That time we have also received a gift from the company BioRad of their newly introduced MicroRotofor™ multicompartment electrolyser. In the work we have studied a free-solution isoelectric focusing (IEF) of protein digests without the addition of carrier ampholytes. In this "autofocusing" mode the tryptic digest itself served as the mixture of ampholytes leading to the separation of the peptides with well-defined pI's. The focusing process was monitored visually using colored pI markers. The resulting fractions were analyzed by CE and electrospray-TOF mass spectrometer using electrospray tips microfabricated in polyimide (Diagnoswiss). Although not all peptides in the protein digests have well defined pI's the autofocusing process can separate many of them leading to higher S/N in the ESI/MS signals and improved protein sequence coverage.

20.

Jarvas, G., Grym, J., Foret, F., Guttman, A. Simulation-based design of a microfabricated pneumatic electrospray nebulizer. Electrophoresis 2015, 36, 386-92.
IF = 2.482; 3 CIT

In this work we have returned to the design of the previously developed microfabricated pneumatic electrospray nebulizer and evaluated two different geometries using computer simulations and experimental measurements of the MS signals. The microdevice was designed for electrospray MS interfacing without the need to fabricate an electrospray needle and can be used as a disposable or an integral part of a reusable system. The design of the chip layout was supported by computational fluid dynamics simulations. The tested microdevices were fabricated in glass using conventional photolithography, followed by wet chemical etching and thermal bonding. The performance of the microfabricated nebulizer was evaluated by means of TOF-MS with a peptide mixture. And the ESI plume shape and stability was monitored by a camera with laser scatter illumination. It was demonstrated that the nebulizer with converging gas channels operated at supersonic speed of the nebulizing gas and produced very stable nanospray (900 nL/min) as documented by less than 0.1% (SE) fluctuation in total mass spectrometric signal intensity.

6. References

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