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# Microfluidic chips for mass spectrometry-based proteomics

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**Microfluidic devices coupled to mass spectrometers have emerged as excellent tools for solving the complex analytical challenges associated with the field of proteomics. Current proteome identification procedures are accomplished through a series of steps that require many hours of labor-intensive work. Microfluidics can play an important role in proteomic sample preparation steps prior to mass spectral identification such as sample cleanup, digestion, and separations due to its ability to handle small sample quantities with the potential for high-throughput parallel analysis. To utilize microfluidic devices for proteomic analysis, an efficient interface between the microchip and the mass spectrometer is required. This tutorial provides an overview of the technologies and applications of microfluidic chips coupled to mass spectrometry for proteome analysis. Various approaches for combining microfluidic devices with electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) are summarized and applications of chip-based separations and digestion technologies to proteomic analysis are presented. Copyright © 2009 John Wiley & Sons, Ltd.**

**Keywords:** microfluidics; proteomics; electrospray; MALDI; microchip analytical procedure

## Introduction

Protein analysis has generated great interest as a means for elucidation of cell function at the molecular level as well as for investigating the relationship between disease states and the protein complement for clinical diagnostics.[1] Electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) are the two most widely used ionization methods for proteomics. One of the main advantages of ESI for proteomic analysis is that it can be used with a flowing liquid, thus facilitating on-line coupling of chromatographic separations to the mass spectrometer. Thus, ESI is an obvious approach for on-line interfacing of microfluidic chips to mass spectrometry. In particular, the nanoliter to microliter per minute flow rate used in microfluidics is a good match to that used in nanoflow ESI sources. Because the typical MALDI analysis requires the preparation of samples containing small crystals of matrix and analyte deposited on a metal target, it is often used in the off-line mode when coupled to liquid separations;[2] fractions from the separation are collected or are deposited directly on the target.[3,4] On-line coupling of liquid separations with MALDI detection is more difficult than with ESI and requires a mechanical, spray, or flowing liquid matrix interface.[5] Coupling MALDI to microfluidic chips can be done with off-line fraction collection or directly from the chip either off-line or on-line.[6–26]

Separation of the components of the proteome is challenging because of the complexity of protein mixtures. For example, it has been estimated that between 100 000 and 250 000 proteins can be encoded by the 20 000 to 25 000 human genes through post-translational modifications and differential splicing, which can produce 5 to 10 different proteins from each gene.[27,28] A further complication is the dynamic range of protein expression at the cellular level, which can range from $10^6$ to $10^9$ copies per cell.[29] Many proteins that are disease biomarkers are low abundant and are difficult to separate from complex mixtures containing highly abundant species with current separation methods.[30,31] As an example, 2-D gel electrophoresis can separate up to 11 000 proteins from a whole cell lysate, but is restricted to the most highly abundant proteins in the sample.[32] High peak capacity separations of proteins with better resolution and faster run times are required for improved proteomic analysis.

Another critical step in proteomic sample pretreatment is the efficient proteolytic digestion of proteins required for reliable identification. Because proteomic samples are typically available in small quantities, efficient digestion protocols are needed to achieve reliable results. Protocols for proteins and cell lysates have been developed for higher digestion efficiency using denaturation steps prior to digestion.[33] In addition, digestion efficiency can be improved through protein enrichment from crude mixtures of cell lysates.[34] Target proteins can be effectively isolated from cytosolic proteins by using affinity beds,[35–37] which selectively capture membrane proteins or through the use of derivatized gold nanoparticles.[38]

Although proteomic analysis methods have advanced significantly in recent years, many analysis steps such as digestion, separation, and other necessary sample preparation steps remain time consuming and labor intensive.[39] Achieving high sensitivity analysis with low sample consumption and high protein sequence coverage are the principal analytical challenges of many proteomic projects.[40] Microfluidic systems have been proposed as a means of automating sequential sample pretreatment steps and also

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as a means to increase sample throughput in parallel proteomic analysis.[41,42] These devices have the advantages of improved portability due to miniaturization, reduced sample and reagent consumption, and accelerated speed of reaction and analysis for high-throughput and highly parallel analysis.[43] In addition, multiple processing steps can be seamlessly integrated to a single chip or multiple chips to allow fully automated sample processing that can address the labor-intensive issues associated with proteomic analyses. Different types of fluidic architectures can be invoked with microfluidic chips to realize unique processing strategies compared to the conventional bench-top approaches that can offer significantly reduced processing times as well. Finally, microfluidic chips can be made from a variety of materials, such as glass or polymers that provide great flexibility in matching the material property to the processing step needs, improving processing step performance.

This tutorial addresses the use of microfluidic devices with mass spectrometry for proteomic applications. An overview of the different approaches toward coupling microfluidic chips to ESI and MALDI is presented. Examples of chip-based separation devices applied to complex proteomic sample mixtures are presented and digestion technologies in microfluidic formats are described.

## Mass Spectrometer Interfaces

Electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) are the ionization methods used most widely for proteomic applications; thus, chip interfaces have focused on these approaches.[44,45] Microfluidic chips can be coupled directly to an electrospray interface using pressure-driven[46,47] or electroosmotic flow[48] to direct the liquid into the spray. Interfacing microchips to MALDI is typically performed off-line through deposition of the chip effluent onto a MALDI target[49] or by ionizing directly from the chip or on-line through a mechanical interface.[19,50]

## Electrospray Interfaces

Several approaches toward coupling microfluidic devices containing electrospray emitters with ESI-MS have been developed. Figure 1 is a schematic diagram of various approaches for the chip ESI interfaces. The simplest approach is to spray directly from the chip (Fig. 1(a)). Here, a glass chip can be scored and broken across a channel for direct spraying from the opening with either pressure or electrokinetic liquid transport used to generate liquid flow.[51,52] More sophisticated spray devices can be fabricated by attaching a capillary to the chip channel (Fig. 1(b)) or by fabricating a spray tip in the device itself (Fig. 1(c)).[6,53] Multichannel microchips can be interfaced to ESI-MS for parallel analysis to increase system throughput.[51]

### Direct from chip

The first interface of a microfluidic chip with mass spectrometry was reported in 1997 with an ESI-MS containing nine microfluidic channels.[51,52] The spray was formed from the blunt edge of a glass chip, which consisted of 60 μm-wide and 25 μm-deep channels connected to a syringe pump generating a pressure driven flow of 100 to 200 nl/min. In the same year, a glass chip was reported with open exits at the end of the channel.[52] The electrospray was generated from the flat edge of the glass channel through an electrokinetically driven flow.

The performance of a direct-from-chip spray is limited by eluent spreading at the interface, which is due to the hydrophilic properties of glass. This ultimately causes difficulty in controlling the spray direction.[48] Moreover, polar liquids wet a large area at the edge of the hydrophilic glass chip, which limits the resolution of separations due to the relatively large dead volume of the electrospray.[54] To prevent spreading of the liquid, the channel exit can be treated to render it more hydrophobic; for example, silanized or polytetrafluoroethylene surfaces have been formed at the edge of the chip to reduce surface-wetting artifacts.[55]

### Attached capillary

An alternate approach to spraying directly from the chip is to attach an electrospray capillary to the chip channel.[56,57] The first approach to this was to use a fused silica capillary inserted into a microchip serving as the mass spectrometer interface.[58] A 12-cm-long fused silica capillary was glued to the edge of the microfluidic chip, and transport of the analyte was accomplished with an electroosmotic pump. Although this work demonstrated the use of capillary emitters for electropspray from a chip, the device was limited to sample infusion and suffered from difficulties in alignment.

A drilling procedure was developed for low dead volume connections between a glass microfluidic chip and an electrospray emitter.[59] The chip had 200 μm holes for emitter attachment produced by 200 μm tungsten carbide bits, producing a 700pl dead volume. The dead volume created by using a conical-shaped carbide bit was effectively removed with a flat-tipped drill bit. Sub-attomole detection limits for peptides was reported by using microfluidic devices with a nanospray tip inserted into a hole perpendicular to the cover of the chip.[6] The tip was of 50 μm i.d. and was pulled to 5 μm i.d. at the tip. Time-of-flight (TOF) MS was used for detection at its maximum acquisition rate of 100 spectra/s. Approximately 0.5 amol of gramicidin S was detected using a 10ms acquisition time period with an observed S/N ratio of 4.

A chip-based integrated liquid junction configuration with a removable electrospray tip was also developed.[60,61] The liquid junction was maintained with a make-up buffer between the cathode end of a capillary electrophoresis (CE) channel and the inlet of the ESI.[62]Approximately 10 pmol of cytochrome *c* was detected using this liquid junction. The junction could provide an effective technique for transferring trace-level samples from a CE separation and improved the spray stability for ESI.[63]

Although capillary spray emitters are more stable, dead volumes in the coupling between the chip and capillary can compromise separation performance and thus liquid separation resolution. Also, attachment of the emitters is labor intensive and potentially a high-cost process for commercial devices. In addition, the glue used to attach the emitter can be dissolved by certain organic solvents and thus produce interference peaks or render the device inoperable.

### Microfabricated sprayer

Electrospray emitter tips from microfluidic chips can be constructed in the microfluidic chip itself.[64] Microfabrication procedures allow for microfluidic channels and emitters to be constructed during chip fabrication and, therefore, many chip components can be made simultaneously. Integrated emitters have

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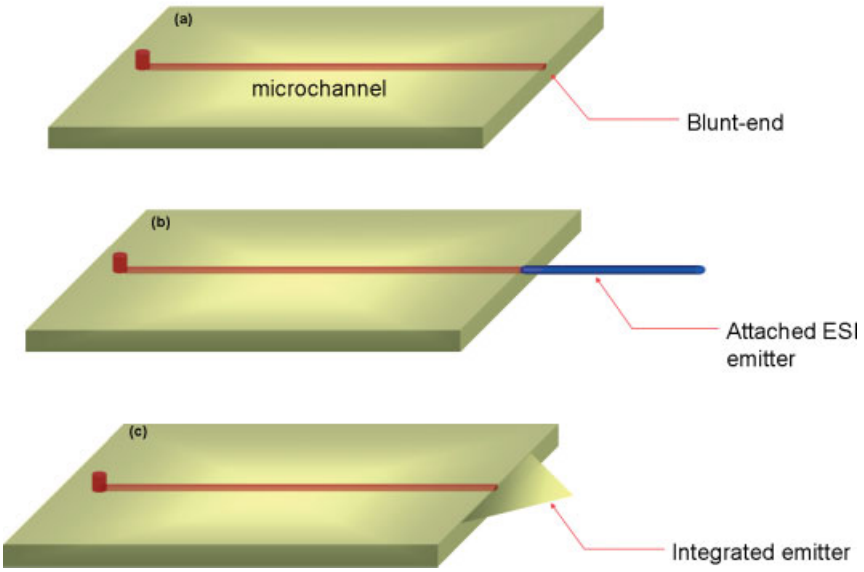


**Figure 1.** Schematic of various microfluidic chip interfaces for ESI for MS using (a) a blunt-end chip, (b) a chip with an attached ESI emitter, and (c) a chip with an integrated emitter.

been constructed on polymer substrates such as polyethylene terephthalate (PET), polycarbonate (PC), poly(methyl methacrylate) (PMMA), and polydimethylsiloxane (PDMS),[65–68] as well as on silicon and glass devices.[69,70] A micromilling machine was used to fabricate an electrospray emitter from PMMA.[65] This method eliminated the dead volume issue between the channel and tip and resulted in a stable spray.

Parylene surface micromachining has also been developed for fabricating integrated ESI emitters.[71–74] In this process, a microchip was fabricated by alternately depositing layers of parylene and photoresist on a silicon wafer. After dissolving the photoresist, the desired parylene structures were formed. Figure 2 shows a photo of the fabricated LC chip. The device had an integrated a pumping system with electrochemical pumps capable of loading a sample and delivering a solvent gradient and a reversed-phase column and ESI nozzle. A mixture of peptides from a bovine serum albumin (BSA) digestion was used to test system performance for proteomic samples. Peptide mass mapping using MS/MS data gave 53% sequence coverage for BSA, which was comparable to 60% sequence coverage for a commercial LC analysis. A gradient sample run was accomplished in approximately 15 s total cycle time, compared to 15 min for the commercial LC system.

A multinozzle chip was developed with an array of hundreds of microfabricated high aspect ratio spray tips formed from silicon using deep reactive ion etching (DRIE).[70] The multielectrospray nozzle chip is now available commercially (Nanomate, Advion, Ithaca, NY).[75] However, no on-chip microfluidic components have been developed.

Another commercial device (Agilent, Santa Clara, CA) has been developed that uses a multilayer polyimide chip with an integrated spray tip formed by laser ablation.[64,76] The chip consists of enrichment and separation channels and an integrated electrospray tip.[77] The microfluidic chip is inserted between a stator and a rotary switching valve. The off-chip valving reduces the chip complexity, but increases the off-chip size and complexity. During operation, the sample is introduced into the enrichment column, which traps the peptides. By switching the valve, trapped peptides are eluted and injected onto the separation column.

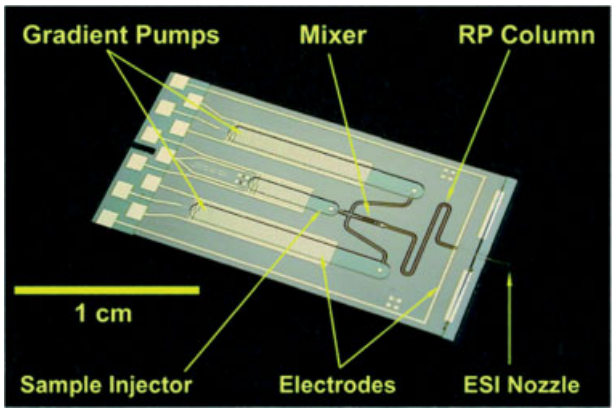


**Figure 2.** Photograph of the microfluidic LC system interfaced to MS. (Adapted from Ref. [74]).

The performance of this microfluidic device coupled to MS is comparable to conventional HPLC-ESI methods. For example, Fortier *et al*. evaluated the analytical performances of the HPLC-chip interfaced with both ion trap and TOF MS for proteomic analyses.[78] This chip was investigated in terms of peak capacity, reproducibility, sensitivity, and linear dynamic range for peptide analysis using digested peptides of complex protein extracts including albumin- and immunoglobulin-depleted rat plasma samples. The peak measurements of more than 626 peptides from a mixture of 8 digested proteins were reproducible over 10 replicate runs. The relative standard deviation in terms of retention time, mass accuracy, and peak capacity were 0.5, 0.003, and 9.1% respectively. Also, this HPLC-chip was used as part of a multidimensional HPLC system for the analysis of the nucleolar proteome, which are involved in cellular processes and are related to several disease states.[77] The HPLC-chip coupled with ion trap MS could identify 151 tryptic peptides in the human colon carcinomal Isreco1. Combined with the HPLC-chip and strong cation exchange chromatography (SCX), 2024 tryptic peptides were identified within the cells.



  

Figure 3 shows representative ESI spectra of a single fraction of collected peptides from SCX using the HPLC-chip. In this figure, (a) a total ion chromatogram of the fraction from the HPLC-chip, (b) MS data of a peak at 26.3 min, and (c) MS/MS of a selected ion of *m/z* 929.3 are depicted. The system has been used for protein identification in a cancer cell line.[79] After extracted proteins from the colon cancer cell line Caco-2 were separated by two-dimensional (2D) gel electrophoresis, the HPLC-chip coupled to ion trap MS was used for identification of the proteins. The sensitivity of the HPLC-chip system was increased fivefold compared to the conventional nano LC/MS method.

## Maldi Interfaces

MALDI is typically carried out on crystallized sample spots containing matrix and analyte, most often under vacuum. Off-line coupling can be accomplished by dropping, spraying, or spotting the sample onto a sample target and adding matrix for analysis.[14,21,26,41,80] On-line coupling of microfluidic devices to MALDI is challenging, but can be achieved using spray,[81–83] continuous flow,[8,17] or mechanical interfaces.[19,84] Of these, only continuous flow and mechanical interfaces have been coupled to microfluidic chips; these are described in detail below.

### On-line MALDI

The continuous flow approach for interfacing microchips to MALDI uses a pressure driven flow of sample in a liquid matrix to bring the mixture into the mass spectrometer for laser ionization.[8,85] This approach was adapted for microfluidic chip analysis using the vacuum of the mass spectrometer to pull the liquid through the chip and into position for analysis.[17] A microreactor chip was constructed from a borosilicate glass wafer comprising two inlet reservoirs, an exit reservoir, and a reaction microchannel with an active volume of 1.2 µl. Reaction reagents were loaded into the two inlet reservoirs that were sealed prior to attaching the chip to a recessed area in the MALDI plate. When the target was inserted into the vacuum chamber, the vacuum-induced flow occurred between closed inlet reservoirs and the open exit reservoir. On-line chip-MALDI analysis was demonstrated for *in situ* chemical reactions, polymer separations, and an oligonucleotide enzymatic reaction.

On-line MALDI can also be accomplished with a mechanical interface that delivers matrix and analyte from the atmospheric pressure separation to the ion source under vacuum.[50,80,84,86,87] The matrix and analyte are deposited on a rotating ball that carries the resulting thin film of matrix and analyte past a vacuum seal and into the MALDI ion source. The coupling of microfluidic separations with the rotating ball interface has been reported by our group.[19,84] Hot-embossed PMMA chips were constructed with a sharp V-shaped tip at the channel exit, as depicted in Fig. 4. The

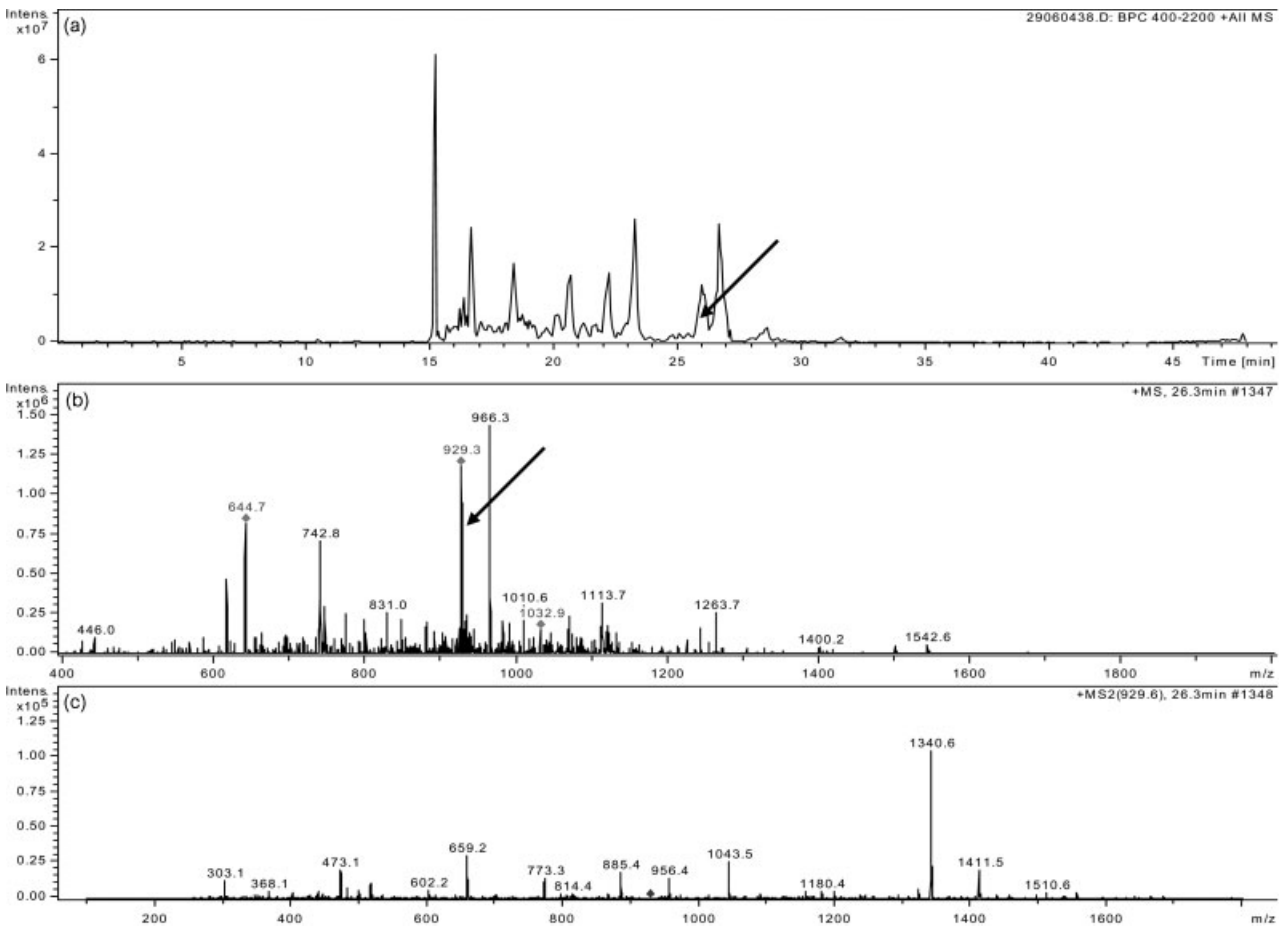


**Figure 3.** (a) A total ion chromatogram of a single fraction from nucleoli using a microfluidic chip; (b) mass spectrum at the peak of 26.3 min indicated by the arrow; (c) tandem mass spectrum of a selected ion of *m/z* 923.9 indicated by the arrow. (Adapted from Ref. [77]).

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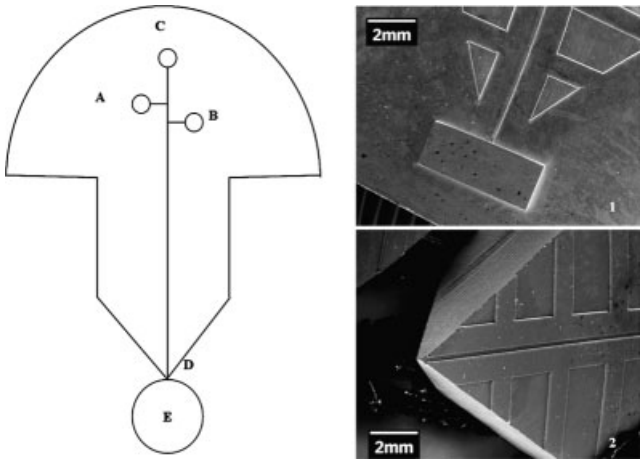


**Figure 4.** (a) Schematic of an on-line microfluidic chip MALDI interface using a rotating ball inlet: A, sample inlet reservoir; B, sample waste reservoir; C, buffer reservoir; D, channel exit; E, rotating ball; Separated samples are deposited onto the ball through direct contact of chip. (b) Scanning electron microscopy images: 1, brass mold master; 2, a hot embossed PMMA chip. (Adapted from Ref. [19]).

tip was positioned against the surface of the ball and the eluent from a CE separation on-chip was deposited as it rotated. The system was demonstrated with the separation of peptides from a tryptic digest of cytochrome *c*. Mass resolution is compromised in the MALDI TOF configuration; however, the system may be adaptable to atmospheric pressure MALDI, which would avoid these problems.

### Off-line MALDI

In off-line MALDI, samples are deposited directly on a target for later analysis in the mass spectrometer. The most direct method for sample deposition is spotting, for example, with a robotic target spotter. Here the matrix is mixed with the analyte before deposition or spotted onto a matrix-coated target. In this manner, fractions from a microcolumn HPLC separation can be collected onto a MALDI target. In one example,[88] the microcolumn was packed with 5-μ m $C_{18}$-modified silica particles. The matrix solution was coaxially mixed with fractions using a capillary mounted at the exit of the separation column. The fraction volumes from the system were approximately 145 nl with a 1 mm spot size. Using this system, peptides contained in single neurons from a snail were fractioned and identified by MALDI-MS.

To limit dead volume, a microfluidic chip can be mounted within the fraction collector. In work from the authors' lab, an automated digestion and deposition system was developed using a microfluidic chip mounted inside a commercial MALDI target spotter.[26] The chip contained a surface-immobilized protein microreactor with a 400 nl volume. Proteins were injected onto the chip using pressure-driven flow, digested in the microreactor and merged with a coaxial matrix flow and spotted onto the MALDI target. Figure 5 shows a schematic of the automated digestion and deposition system. The time for protein digestion was less than 1 min for protein digestion, mixing with matrix and deposition. A typical MALDI mass spectrum tryptic digestion of cytochrome *c* using the microreactor is shown in Fig. 6. The 10 μM solution of cytochrome *c* was infused into the bioreactor at a flow rate of 1 μl/min, which afforded a residence time within the reactor of 24 s. Asterisks in this figure indicate matched peaks from peptide mass mapping. As seen in Fig. 6, fragments containing 70 out of the 104 possible amino acids of cytochrome *c* were obtained. In our search using peptide mass lists of cytochrome *c*, 67% sequence coverage with 137 Mowse score was achieved.

The primary difficulty with target spotting is the relatively large volume contained in a droplet from a small diameter capillary. For example, a 50 μm capillary produces droplets that are approximately 300 nl, which can be a significant volume compared to peak volumes emanating from microfluidic separation components. It can also be difficult to control the spotting volume with high precision. To overcome these problems, piezoelectric dispensing of samples onto the MALDI target can be used.[89] A piezoelectric droplet dispenser has been developed for interfacing a microfluidic chip to a high-density array of nanovials etched into a silicon MALDI target.[41] The nanovials were 300 μm square and 20 μm deep and served to collect and concentrate the sample by multiple droplet depositions on the same spot. Figure 7 shows the microfluidic chip system with the piezoelectric microdispenser interface. The device generated droplets ranging from 65 pl to 300 nl at a droplet frequency of 50–100 Hz. The system was demonstrated with peptides generated from a chip tryptic-based microreactor.

An alternate sampling approach is electrospray deposition onto a MALDI target. Several studies have demonstrated the viability of electrospray deposition of eluent from CE, HPLC, and size-exclusion chromatography (SEC) onto the MALDI plate.[90] The eluent is delivered to the electrospray capillary operated at 2–4 kV and the spray is directed onto a MALDI target several centimeters away. Rapid solvent evaporation from the small droplets leads to homogeneous spots on the target. A hydrophobic membrane electrospray deposition device was developed for interfacing polycarbonate microfluidic chips to MALDI.[21] The system, shown in Fig. 8, consisted of a 50 μm-thick polytetrafluoroethylene (PTFE) membrane thermally annealed to the outlet of a PC microfluidic chip. The PTFE provided a hydrophobic spray surface, and a T-junction was used for the electrical contact for the electrospray and the analyte solution was flowed through the chip with a syringe. Multiple electrospray tips deposited parallel sample spots spaced 150 μm apart on the MALDI target.

In some cases, the chip itself was used as a MALDI target. A spinning compact disc (CD) format was employed for microfluidic sample treatment prior to off-line MALDI directly from the chip.[12] Fluid flow through the chip was controlled by disk rotation speed. In one configuration, samples were delivered by centrifugal force through a 10nl column packed with 15 μ m $C_{18}$ beads. After desalting and washing, a solution containing the matrix was used to elute sample from the column and onto a gold-coated MALDI target region. The chip was attached to a MALDI target holder and analyzed in the mass spectrometer. This CD format (Gyros AB, Uppsala, Sweden) is now commercially available for cleanup and isolation of proteins. One application focused on the identification of vascular endothelial proteins using a CD microfluidic system containing hydrophobic resins packed in each column.[91] Combined with 2D gel electrophoresis, this CD chip provided high yield in both Coomassie stained and Sypro Ruby stained proteins. The chip could analyze 48 samples simultaneously from in-gel digestion of proteins. Compared to typical ZipTip purification, the CD chip offered higher sequence coverage. This CD microfluidic technology was also used for sample purification of real samples from patients with colon cancer.[92] Relative proteome alterations in expression levels between normal



  

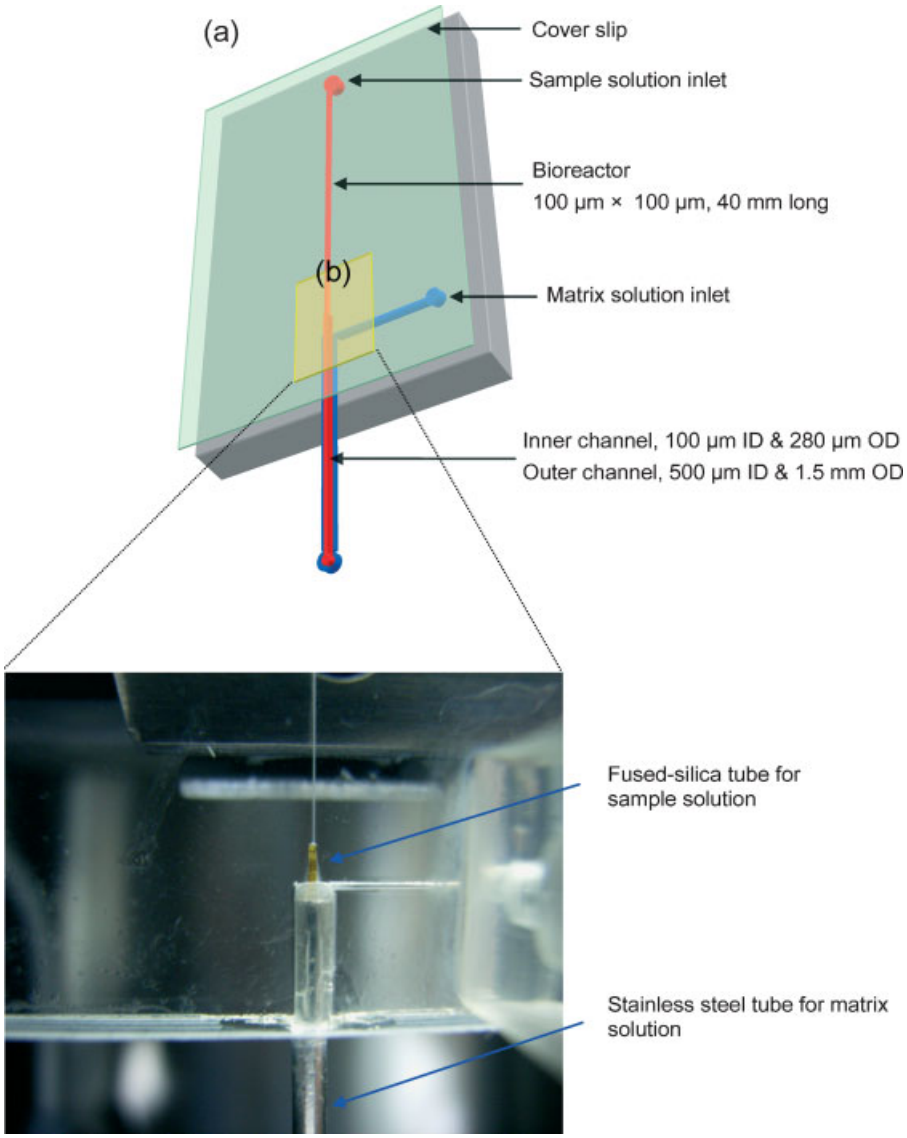


**Figure 5.** Schematic of an automated digestion and deposition system for MALDI. The system contains a microreactor measured 40 mm × 100 × 100 μm and a matrix solution channel. On the end of the microreactor, coaxial tubes are sealed to mix typtic digested peptides with a matrix solution and to deposit onto a MALDI target plate. (b) Assembled microreactor: chip components including PMMA chip and cover slip, inlet fused-silica tube and stainless steel tube for inlet and outlet connectors, respectively. (Adapted from Ref. [26]).

tissue and carcinoma were evaluated both inter- and intra-patient. The detection of 112 protein changes at the proteome level, and identification of 72 of these proteins were achieved using 2D gel electrophoresis combined with CD microchip MS. Instead of desalting by reverse-phase chromatography an immobilized metal affinity chromatography (IMAC) microfluidic column was used.[93] Enriched and peptides eluted, phosphopeptides could be enriched using this chip and peptides eluted from the columns directly onto a MALDI target plate. The success rates for phosphopeptide analysis were over 90% with femtomole level detection limits for model peptides, which were $\beta$-casein tryptic phosophopeptides, phosphotyrosine peptide, and BSA tryptic peptides.

In the authors' lab, a capillary gel microfluidic chip was developed for direct analysis using a pulsed infrared laser and no added matrix.[25,94] This gel microfluidic chip interfaced to matrix-free IR laser desorption/ionization (LDI) allowed the detection of peptides and proteins at a significantly improved detection limit compared to analysis with conventional gel slabs. Further, the addition of dyes was not needed for visualization of spots due to the reduced size and dimensions of the gel. This chip was fabricated from PMMA with a PDMS cover slip and a cross-linked gel was introduced into the microchannels by polymerization. After gel electrophoresis, the cover slip was removed and either the chip or the cover was mounted onto a modified MALDI target holder for analysis. The channel was irradiated with 2.95 μm radiation from a pulsed optical parametric oscillator (OPO), which was coincident with IR absorption of the gel and water within the gel. The IR laser has a greater depth of penetration compared to the UV laser, allowing removal of material from the entire gel channel, thus enabling improvement in the detection limit. Although a separation of the bradykinin and bovine insulin was accomplished in the gel microfluidic chip, there was significant band spreading of analytes over the microchannel due to interactions between analytes and the channel walls.



  

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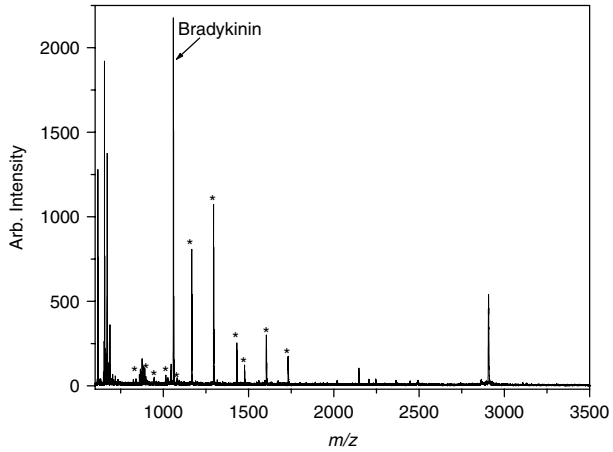


**Figure 6.** MALDI mass spectrum of a tryptic digest of cytochrome *c* at 60 s using an automated digestion and deposition microfluidic system: 10 µm in 50 mM ammonium bicarbonate buffer; flow rate, 1 µl/min; deposition time, 20 s (3.3 pmol); asterisks indicate matched peaks. Bradykinin is an internal standard.

## Sample Purification

A sample is typically purified to remove salts and buffers and concentrate the sample prior to analysis by mass spectrometry, and some of these procedures have been adapted to chip formats. An on-line desalting method for ESI mass spectrometry has been developed.[95] In this device, a hydrophobic poly(vinylidene difluoride), PVDF, membrane was integrated into an inlet channel of a polyimide chip. This membrane was used for capturing target analytes and, after removing salts by washing with water, a methanol/water solution was pumped into the chip to elute the retained analytes. The cleanup of drugs, peptides, and proteins was demonstrated, and it was found that the background was comparable to salt-free solutions.

A unique device for MALDI sample cleanup used the electrowetting-on-dielectric (EWOD) technique.[22] The EWOD microfluidic chip consists of a bottom plate containing fabricated electrodes beneath a Teflon dielectric layer and an enclosing cover plate. The applied field causes the droplet to move in the channel and can be used to transport liquids in a microfluidic device. The EWOD technique was used to purify samples for proteomic analysis. First, sample droplets containing peptides or proteins were moved to specific locations by applying consecutive electrical potentials. Next, a water droplet was moved over the deposit to dissolve impurities and then removed. Finally, a solvent droplet with matrix was delivered to the sample spot and dried. MALDI was then performed on the dried sample spot. The EWOD approach was found to be more efficient at sample cleanup compared to conventional approaches. For example, insulin and angiotensin II solutions with 8 M urea could be cleaned using the EWOD process to yield an increase in the S/N ratio compared to when not treated.

## Separation

Microfluidic chip separations for proteomics uses either CE or liquid chromatography (LC) with packed beads or porous monolith stationary phases.[69,96,97] CE is suited to microfluidic chip formats due to fast separation times and the ability to drive the flow with an electric field rather than a mechanical pump. With LC, the microfluidic channels typically must contain packed beds or monoliths to reduce the diffusion distance for efficient separations.

### Capillary electrophoresis

There have been a number of approaches to coupling chip-based CE separations to mass spectrometry.[98–100] These have used different variants of CE, such as capillary gel electrophoresis (CGE),[101,102] capillary zone electrophoresis (CZE),[103] micellar electrokinetic chromatography (MEKC),[104] and isoelectric focusing (IEF).[105] An electrophoresis separation of biological mixtures in open channels has been coupled off-line with MALDI-MS.[85] The chip was made of glass with 250 µm-wide and 250 µm-deep channels. After the CE separation of oligosaccharides and peptides, the chip was placed on a moving stage installed inside the source chamber for MALDI analysis directly from the chip.

Recently, a microfluidic chip was developed for CE separations coupled to ESI MS.[69] The device was made from a borosilicate glass substrate with a 75 µm-wide and 10 µm-deep separation channel. The electrospray was generated at the corner of the chip. Figure 9 shows a schematic of two such chips, one with a straight 4.7 cm channel and the other with a serpentine 20.5cm-long channel. The channel surfaces were coated with polyamine to minimize surface interactions and enhance electroosmotic flow. Peptides and proteins were tested for on-chip CE separation coupled to MS. The separation efficiency of this chip was about 200 000 theoretical plates for the 20.5cm-long channel with a calculated peak capacity of 43. The separation was accomplished in less than a minute to several minutes, depending on the channel length.

Several technical challenges remain for on-chip CE separations.[106] For example, separation efficiency can be reduced due to the interaction of analytes with chip surfaces. Additionally, the injection volume is limited for CE microchip separations, necessitating a sample-enrichment unit such as solid-phase extraction prior to injection for some proteomic applications directed toward analyzing low abundant proteins.[107] Further, there remains the problem of the compatibility of the reagents used for the on-chip separations with the on-line electrospray. A liquid junction or sheath flow may be required to couple on-chip CE with ESI-MS because separation buffers and other reagents used for the separation may not be compatible with ESI.[56]

### Liquid chromatography

LC capillary columns have inner diameters in the range of 75 to 500 µm that are similar to the sizes of microfluidic channels and, therefore, separation protocols can be readily adapted to a chip format.[108] Chip-separation channels have typically under 300 µm internal diameters and are noncylindrical, for example, square, rectangular, or semicircular, and are fabricated in silicon, glass, quartz, or polymer.[109] The stationary phase can be formed using packed beads or on monolithic support formed by *in situ* polymerization.

Packed-bead chromatography inside a microchannel is similar to conventional HPLC, thus many established concepts can be directly applied to microfluidic systems for proteomic analysis. However, packing a microchannel with beads is a technically difficult process owing to the resistance to fluid flow, which can result in uneven packing and voids within a column.[110] The high pressure in the channels can cause leaks or bubbles and it can be difficult to introduce frits or other structures to trap the beads and prevent their movement through the channels.



  

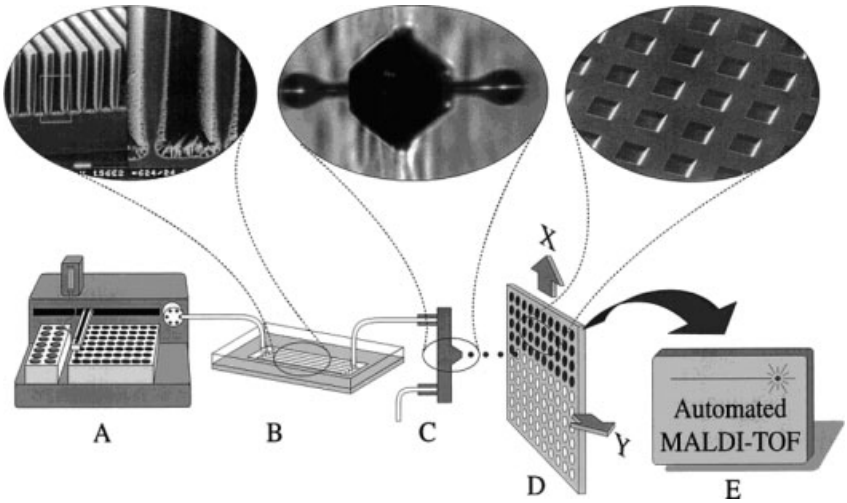


**Figure 7.** Schematic of total analysis system using a piezoelectric deposition interface for MALDI-MS: A, sample pretreatment; B, microreactor; C, piezoelectric flowthrough dispenser; D, MALDI target plate consisting of 300 × 300 × 20 μm nanovials; E, MALDI-TOF MS analysis. (Adapted from Ref. [41]).

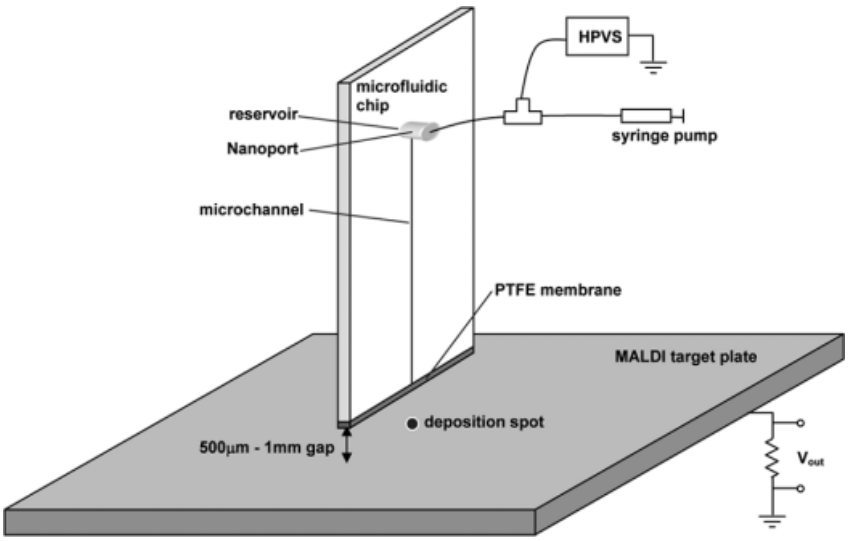


**Figure 8.** Schematic of an electrospray deposition interface for MALDI. The system consisted of a PTFE membrane thermally bonded to the outlet of a polymer microfluidic chip, a T-junction for providing high voltage, and a syringe pump for delivering analyte solution. The electrospray gap was controlled by a multiaxis stage. (Adapted from Ref. [21]).

To create a packed stationary phase for microfluidic chips, the channel can be filled with beads having diameters between 3 and 5 μm and pore sizes selected according to the analyte and required separation efficiency. For example, 3.5-μm $C_{18}$ beads with 30 nm pore size were used to pack a chip-separation channel, which had 75 μm width, 50 μm depth, and 45 mm length.[64] This chip was demonstrated for the separation of tryptic peptides of BSA with on-line ESI-MS analysis. The retention times for tryptic peptide peaks from an extracted ion chromatogram were reproducible over three runs with less than 0.1 min standard deviation. Recently, the relation between the packing density and separation efficiency of packed-bed microchips was investigated.[96] A custom-built stainless steel holder and ultrasonication were used for high packing density. The separation channel had a 75 μm × 50 μm trapezoidal cross section and the packing material used was 5-μm $C_{18}$ with an 80 Å pore size. These optimized packing procedures resulted in separation efficiencies for tested organic compounds comparable to those of commercial nano-HPLC.

### Monolithic supports

Monolithic supports for HPLC stationary phases were first introduced 20 years ago and have emerged as an alternative to packed columns because of the simplicity of *in situ* polymerization.[111–113] These columns are completely filled with porous material and thus have no interparticle voids, which allows all of the mobile phase to flow through the pores of the stationary phase. Aside from these merits, monolithic columns have several advantages over packed columns for microfluidic chip applications, including simple modification of porosity, surface area, and functionality and the ability to form the column in place without the need for retaining frits.[114] Disadvantages are the limited chromatographic media[64,114] and difficulties in producing homogeneous supports due to large difference in polarity of monomers.[115,116]

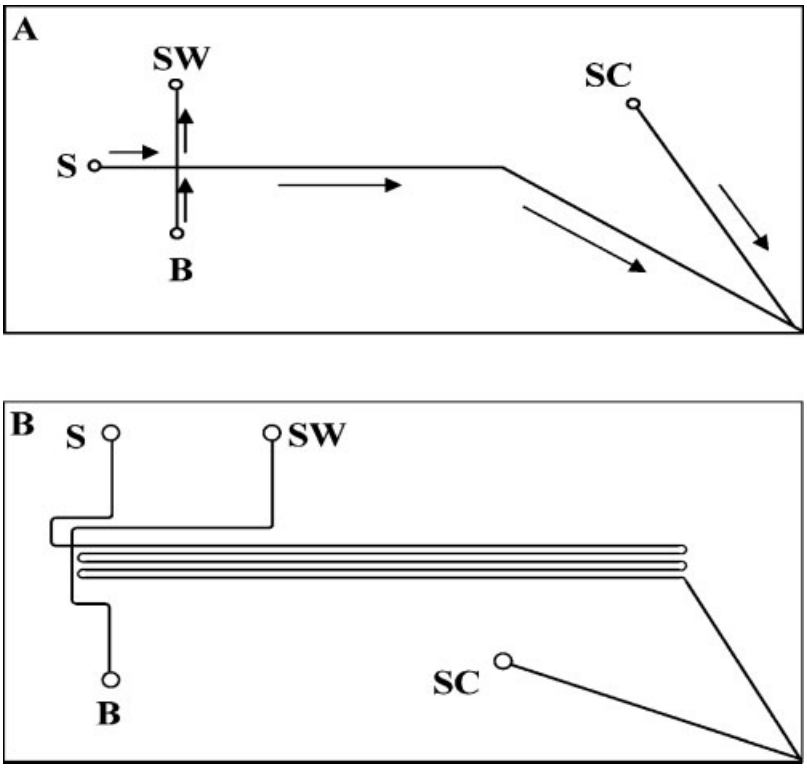


**Figure 9.** Diagrams of CE-chips for ESI containing short-channel (a, 4.7 cm) and long-channel (b, 20.5 cm) with 75 μm width and 10 μm depth: S, sample reservoir; B, buffer reservoir; SW, sample waste; SC, side channel. The arrow indicates the electroosmotic flow direction. (Adapted from Ref. [69]).

Polymer and silica-based monolithic columns have been developed for microfluidic separations in proteomics;[117] the former has shown excellent properties for large molecule separations, whereas the latter is desirable for the separation of small molecules. A cyclic olefin copolymer (COC) microfluidic device coupled off-line to MALDI-MS has been reported.[118] The chip was prepared with an array of methacrylate monolithic columns that were formed by UV-initiated polymerization. For peptide mass mapping of digested BSA, a 59% sequence coverage was obtained using the on-chip separation device with MALDI-MS analysis compared to 23% sequence coverage without separation. The chip was tested for separation efficiency and reproducibility of peptide mixtures in each column. A sensitivity test showed that a target peptide at 10 fmol/μl could be identified in the presence of 10 pmol/μl digested BSA.

Another interesting chip-based separation has been developed for direct MALDI analysis of separated proteins.[119] A capillary IEF chip sealed with removable resin tape was coupled to MALDI-TOF MS without degrading IEF resolution. The glass chip consisted of four meandering channels, which were approximately 400 μm wide, 10 μm deep, and 60 mm long. After sealing the channels with resin tape, the proteins were separated by IEF and were immediately frozen in the chip to prevent diffusion. The resin tape was then removed and the samples were freeze-dried. Finally, a matrix solution was applied to the channels, and the chip was mounted into a MALDI-TOF MS. The mass spectra were obtained for each 500μm increment of the channel and plotted on a 2D map. Because the glass chip was covered with removable resin tape, it was easy to apply the matrix solution to the separated samples on the chip as well as prevent sample solution from drying out. Compared to the heat-drying process to dry out a sample solution, the freeze-drying method prevented a redistribution of the sample solution in the channel during evaporation.

Monolithic supports for thin-layer chromatography (TLC) coupled with MALDI-TOF MS were developed.[115] A thin poly(butyl methacrylate-co-ethylene dimethacrylate) layer 50 to 200 μm thick was prepared by UV-initiated polymerization. Four different proteins, insulin, cytochrome *c*, lysozyme, and myoglobin, were reproducibly separated and identified by off-line MALDI-TOF MS over five different TLC plate runs. The separation was achieved in 6 min.

## Enzymatic Digestion

Efficient digestion is an essential tool for protein identification with bottom-up proteomic strategies.[120,121] Three different approaches can be used for proteolytic digestion; in-gel,[122] in-solution,[33] or solid phase.[123] In-gel digestion is accomplished by cutting spots from 2D gel electrophoretic bands that contain the proteins of interest, which are then subjected to *in situ* digestion.[124] Drawbacks to this method are limited accessibility to the proteins inside the gel,[125] and gel-destaining procedures can cause poor digestion yields due to residual destaining solvents.[126] Further, the process cannot easily be moved to microfluidic chip formats to realize process automation. A second approach is to digest proteins in solution. This approach requires long incubation times due to the need for low proteolytic enzyme concentrations to minimize autodigestion artifacts and the need to run with relatively high temperatures to achieve high digestion efficiencies.[127] Excessively high temperatures, enzyme concentrations, or reaction times can lead to autolysis of trypsin, nonspecific cleavage, and deamidation.[127–129]



  

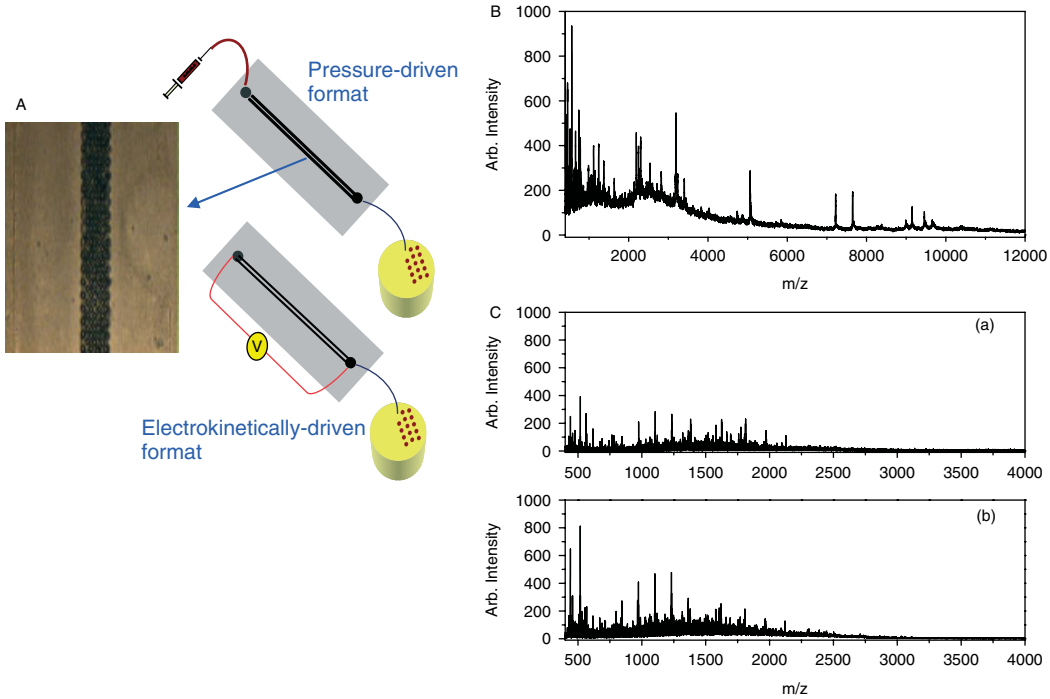


**Figure 10.** (a) Schematic of a micropost bioreactor fabricated by photolithography. (b) MALDI-TOF MS spectrum of intact *E. coli*; 10 mg/ml in 50 mM ammonium bicarbonate buffer (pH 8.2); deposition time, 10 s. (c) MALDI-TOF mass spectra of a tryptic digest of intact *E. coli* using the micropost bioreactor; (a) pressure-driven flow format; flow rate, 1 μl/min; (b) electrokinetically driven flow format; field strength, 375 V/cm. 10 mg/ml in 50 mM ammonium bicarbonate buffer (pH 8.2); deposition time, 10 s.

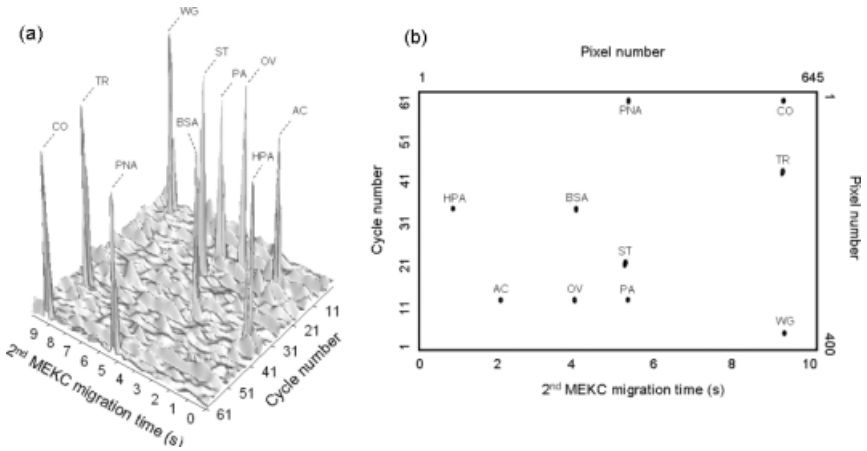


**Figure 11.** A 2D electrophoretic separation of a 30 nM protein mixture using a PMMA microfluidic chip; the first dimension of separation is SDS $\mu$-CGE and the second dimension of separation is MEKC. Panel (a) shows a 3D image of the data for cycle number versus the MEKC migration time and (b) shows a converted 2D image of (a) similar to a 2D stained gel. (Adapted from Ref. [151]).

Solid-phase digestion uses proteolytic enzymes chemically immobilized or adsorbed on the surface of a solid support.[130,131] This digestion protocol has the advantages of fast response, low sample consumption, and high throughput,[132] and is easily adaptable to microfluidic chip formats. A solid-phase microreactor minimizes sample loss during treatment and reduces autolysis products of proteolytic enzymes;[133] the short diffusion distance for properly designed reactors, and a high enzyme-to-substrate concentration ratio results in higher digestion efficiencies compared to in-solution digestion.[47]

The digestion efficiency for on-chip formats depends on the geometry of the reaction chamber, the temperature, the solvents used, and the applied voltages for electrokinetic transport.[7,24,47,134] Organic solvents can improve digestion efficiency by denaturing the proteins; however, this can be a disadvantage for solid-phase digestion and can damage the immobilized enzyme with the solvent.[135] Thermal and electrical denaturation are relatively free of contamination because additional reagents are not added.[136,137] However, these methods require additional control systems for temperature and voltage, which increases the overall chip complexity.

The geometry of the microreactor can be in an open channel or a three-dimensional (3D) structure. An open channel format is the simplest configuration, but digestion efficiency is limited by the relatively long diffusional distances, which can limit the digestion rate. Also, diffusion rates of proteins depend on their

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concentrations as well.[138] Lower diffusion rates are expected at higher concentrations of proteins in an open channel, thereby achieving low digestion efficiency due to limited encounter numbers between proteolytic enzymes and proteins. A 3D solid-phase reactor format can be configured from a monolithic porous network,[139,140] or a packed channel.[141] The high surface-to-volume ratio compared to open channel increases the digestion efficiency due to the lower diffusional path length, which allows for more encounters between the substrate and immobilized enzyme.[20] A microfluidic chip packed with trypsin-derivatized beads in a fluidic channel has been reported.[141] The bead-packed chip provided faster protein digestion and fewer trypsin autolysis products compared to a homogeneous digestion.

Solid-phase microreactors can be formed *in situ* by immobilization of enzymes through covalent attachment to supports or encapsulation within gel matrices.[123,139,140,142] This avoids the difficulties arising from packing beads into the microchannels. Monolithic supports for immobilizing proteolytic enzymes can be generated through a polymerization reaction of a monomer solution into a microfluidic channel.[143] For example, a porous organic polymer monolithic microreactor was developed in which trypsin was immobilized within the monolith using azlactone functional groups for covalent attachment of the enzyme.[139,142] The sequence coverage of tryptic peptides from myoglobin was determined by off-line MALDI and was found to be 67% for a 12 s residence time. A silica sol–gel monolith containing zeolite nanoparticles has been reported.[123] This device had a high surface area for the immobilized enzyme, allowing for a high load level within the microreactor. A 0.5 μl volume containing 0.2 μg/μl of the proteins cytochrome *c* and BSA were digested within 5 s in the microreactor as indicated by off-line MALDI-TOF MS. The bioreactor could be used repeatedly and the enzyme remained active for more than a month when it was stored at temperatures below 4 °C. A pepsin microreactor was developed using a sol–gel monolithic column photo-polymerized within a fused silica capillary.[140] The column was used for on-line ESI CE/MS. Although monolithic microreactors are fast and efficient, it can require more than 24 h for their preparation and it can be difficult to make them reproducibly.

A 3D microreactor can be created using microstructures within the channel, which bypasses problems associated both with bead packing and with monolith formation in the channel.[144,145] The channel structure is fixed, for example, by the mold master when used to replicate a polymeric device, which provides a reproducible microreactor. Furthermore, the support structures are placed in a desired location within the device and can provide unrestricted substrate access to the immobilized enzyme.

In the authors' laboratory, a 3D microreactor was prepared with a trypsin immobilized PMMA microfluidic chip containing a micropost structured channel. The solid-phase microreactor consisted of a 4 cm-long, 200μm-wide, and 50 μm-deep microfluidic channel with an array of 50 μm-diameter micropost support structures with a 50 μm inter-post spacing. The microfluidic chip was fabricated by hot embossing with a brass master that was created with a high-speed micromilling machine. Trypsin was covalently immobilized onto the surface of the microposts using UV-mediated surface-modification protocols.[144] This system employed an electric field of 375 V/cm to transport the peptides from the enzymatic bioreactor directly onto a MALDI target by blotting deposition. Cytochrome *c* was used as a model protein for testing the performance of the micropost bioreactor. For a 20s residence time, 19 fragments containing 101 out of the 104 possible amino acids of cytochrome *c* were identified, corresponding to a 97% sequence coverage. This compares favorably with the 67% sequence coverage that was obtained from an open-channel microreactor under similar reaction conditions.[26]

Photolithography was used for creation of a more complex master with a higher surface-to-volume ratio. The chip consisted of a 3.5 cm-long, 230 μm-wide, and 100 μm-deep microfluidic channel with trypsin immobilized onto an array of 30 μm × 30 μm, diamond-shaped microposts with a 20 μm edge-to-edge inter-post spacing (see Fig. 10). This micropost bioreactor was used for on-chip enzymatic digestion of *Escherichia coli* (*E. coli*, ATCC 9637) bacteria to demonstrate the approach. A solution containing *E. coli* suspended in 50 mM $NH_4HCO_3$ was injected into the bioreactor using pressure-driven or electrokinetically driven flow. In the pressure-driven mode, sample solution was infused at flow rate of 1 μl/min and was stopped for 5 min to allow time for digestion. For the electrokinetic flow, a field strength of 375 V/cm was applied into a sample inlet reservoir and an outlet reservoir for 1 min, and the sample was retained for 5 min in the reactor. After digestion, the sample was collected on a MALDI target. A MALDI mass spectrum of intact *E. coli* is shown in Fig. 10(b). In this figure, several intact proteins, which were dissolved in ammonium bicarbonate buffer, can be found. Figure 10(c) shows representative MALDI mass spectra obtained with pressure-driven and electrokinetically driven flow modes. As can be seen in this figure, proteolytic peptide peaks were found. On-chip digestion with electrokinetically driven flow resulted in greater peak intensities compared to pressure-driven flow.

## Integrated Proteomics Chips

A proteomics chip is a fully integrated microfluidic platform that combines multiple functions on the same chip, providing complete proteomic analysis and, thus, fully automated analysis. Most of the recent developments in microfluidics for proteomic applications in mass spectrometry have focused on developing chips with a single functional element and optimizing the mass spectrometer interface. Although fully integrated proteomic analysis chips do not yet exist, there have been several developments toward the integration of multiple processing steps into chips.

Two-dimensional separations are extremely important for the realization of integrated microfluidic systems employed for complex proteomic samples. For example, the on-chip combination of CEC-CE, MEKC-CE, and IEF-CE has been reported for high peak-capacity 2D separation.[146–148] Recently, a PMMA-based microchip was developed for the high-peak capacity 2D electrophoretic separation of proteins with a protein band volume on the order of ~10 pl.[149] The separation modes for the first and second dimensions were sodium dodecyl sulfate microcapillary gel electrophoresis (SDS $\mu$-CGE) and micellar electrokinetic chromatography (MEKC) respectively. Figure 11 shows a 2D separation of a 30 nM protein mixture in the PMMA microchip. A peak capacity of approximately 1000 was achieved for this 2D chip separation with 10 proteins that were completely separated within 12 min. To date, 1000 to 4000 peak capacity has been achieved with this novel 2D microfluidic separation.[147,149] In order to be utilized for proteomic purposes, a peak capacity of more than 10 000 will be needed to be comparable with conventional 2D-PAGE.[108] Direct combinations of 2D separation chips with mass spectrometry are under development. In order to adapt 2D microfluidic separations with ESI or MALDI, the utilization of the



  

small sample volumes must be optimized. For example, MALDI sample deposition with small deposited spot sizes for a higher local concentration may be necessary.

To adequately address the current limitation of proteomics analysis, multiple processing steps must be employed. For example, microfluidic devices integrating the processing steps of sample isolation and extraction, solid-phase digestion, and separation prior to MS detection are particularly attractive. These devices must be capable of handling the limited quantities of proteins in cellular extracts for clinical diagnosis, ideally at the single-cell level. Further up-stream proteomic sample preparation integration such as cell isolation, culturing, and cell lysis is another developing area. These steps can be combined on the same device for highly parallel automated analysis, which is particularly compelling for high-throughput proteomic processing.

## Conclusions

Although there are many remaining challenges for adapting and integrating the necessary sample processing steps such as purification, separation, and digestion to microchip platforms, microfluidic devices are approaching the stage where they can become important tools for proteomic analyses. A number of strategies for coupling on-chip separation and digestion techniques with MS analysis have been developed. ESI interfaces have undergone improvements for the past decade and have evolved from simple, blunt edge sprayers to inserted, fused-silica tips and integrated microfabricated emitters. For MALDI interfaces, several on-line and off-line approaches have been developed. On-line coupling of microfluidic chip separations with MALDI is challenging but can permit the direct acquisition of sample information and has the potential for high-throughput through multiplexing. Off-line approaches to coupling microfluidic chips with MALDI have been reported that use target deposition systems or integrated MALDI target chips. Several types of microfluidic chip devices have been developed as commercial systems for proteomics research. Future trends in microfluidics for proteomics will address the need for robust and reliable interfaces between microfluidic devices and MS. Furthermore, the integration of microfluidic components for an all-in-one microfluidic chip for mass spectrometry–based proteomics remains an important goal.

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