MICROFABRICATED DEVICES: A NEW SAMPLE INTRODUCTION APPROACH TO MASS SPECTROMETRY

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Instrument miniaturization is one way of addressing the issues of sensitivity, speed, throughput, and cost of analysis in DNA diagnostics, proteomics, and related biotechnology areas. Microfluidics is of special interest for handling very small sample amounts, with minimal concerns related to sample loss and cross-contamination, problems typical for standard fluidic manipulations. Furthermore, the small footprint of these microfabricated structures leads to instrument designs suitable for high-density, parallel sample processing, and high-throughput analyses. In addition to miniaturized systems designed with optical or electrochemical detection, microfluidic devices interfaced to mass spectrometry have also been demonstrated. Instruments for automated sample infusion analysis are now commercially available, and microdevices utilizing chromatographic or capillary electrophoresis separation techniques are under development. This review aims at documenting the technologies and applications of microfluidic mass spectrometry for the analysis of proteomic samples.
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I. INTRODUCTION

The ability to perform mass spectrometric analysis for the characterization of low-level protein expression in isolated cells or tissue fragments is highly dependent on the effectiveness and efficiency of the initial sample preparation steps. Whether traditional 2D-gel electrophoresis protein separation protocols or alternative approaches that involve batch processing and digestion of total cell lysates are utilized prior to mass spectrometric analysis, the overall procedure involves numerous steps that are usually performed sequentially, are time-consuming and labor intensive. Sample enrichment and separation are especially important for the investigation of mixtures with hundreds or thousands of components (e.g., protein digests) where analyte signal suppression can be a serious issue. Parallel processing and automation could significantly reduce analysis times; however, these features become feasible only if adequate high-throughput instrumentation and protocols are made available.

This past decade has witnessed significant progress in the field of microfluidics, instrument miniaturization, and analytical process integration. Microfabricated devices and ''Lab-on-a-Chip'' technologies (Manz, Graber, & Widmer, 1990a; Manz et al., 1990b, 1991; Harrison et al., 1992; Harrison, Glavina, & Manz, 1993; Jacobson et al., 1994a,b,c), which possess the ability to control the manipulation, distribution, and detection of minute amounts of sample (Murakami et al., 1993; Jacobson et al., 1994b; Woolley & Mathies, 1995; Jacobson & Ramsey, 1995a; Jacobson, Moore, & Ramsey, 1995b; Madou, 1997; Martynova et al., 1997; Roberts et al., 1997; Ford et al., 1998; Duffy et al., 1999; McEnery et al., 1999; Xu et al., 2000; Bousse et al., 2001), are expected to play an important role in the development of high-throughput instrumentation. The major features associated with miniaturization reside in high-speed, reduced sample volume and reagent consumption, integration of operational elements, and high-throughput capabilities via parallelization. The utility of multiplexed microfluidic devices with mass spectrometry (MS) detection is often argued, as sampling continues to occur in a sequential manner if only one mass spectrometer is used as a detector. During the past years, however, there have been significant efforts invested in developing miniaturized and multiplexed mass spectrometers (Kornienko et al., 1999; Badman & Cooks, 2000; Berkout, Cotter, & Segers, 2001; Tabert et al., 2003), and the interfacing of the two multiplexed systems is anticipated to happen in the near future. In addition, overall throughput should be contemplated with regard to the entire sample preparation effort, and integration will always be a favorable factor for speeding up the analytical sequence of operations. The ability to perform parallel analysis on highly integrated microfluidic devices with multiplexed detection strategies will clearly address the biotechnological need for high-throughput sample processing.

The microchip integration of capillary electrophoresis (CE), capillary electrochromatography (CEC), and micro-liquid chromatography (μLC) separation strategies (Harrison et al., 1992; Harrison, Glavina, & Manz, 1993; Jacobson et al., 1994a,b,c; Lazar et al., 2005), of preconcentration and microreaction elements (Murakami et al., 1993; Jacobson et al., 1994d;

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Jacobson & Ramsey, 1995a), and the coupling of microfabricated devices to electrospray ionization mass spectrometry (ESI-MS), has already been accomplished (Figeys, Ning, & Aebersold, 1997; Ramsey & Ramsey, 1997; Xue et al., 1997a,b). The development of accurate sample injection strategies on the chip (Harrison et al., 1992; Jacobson et al., 1994a), of ultra high-speed CE separations (Jacobson et al., 1998), and of complex 2D-separations, such as micellar electrokinetic chromatography followed by CE (Rocklin, Ramsey, & Ramsey, 2000) or CEC followed by CE (Gottschlich et al., 2001), and the implementation of high-throughput microchip sample analysis (Emrich et al., 2002) represent some major milestones in microfluidic design and performance. The routine use of microfluidics in the analysis of DNA, proteins, peptides, or drug screening (Woolley & Mathies, 1995; Figeys, Ning, & Aebersold, 1997; Xue et al., 1997b, 1998; Figeys & Aebersold, 1998a; Figeys et al., 1998b,c; Li et al., 1999; Xiang et al., 1999) has established some landmarks that fully demonstrate the power of these microfabricated analytical platforms. These devices are capable of handling sample volumes in the microliter range and enable the analysis of volumes as low as 1–10 pL. Typical sample manipulations, such as injection, separation, labeling, and detection, can be performed in a few minutes or seconds. Jacobson et al. (1998) has demonstrated a high-speed CE experiment on a microchip that involved the separation and detection of two fluorescent components in a 0.8-msec timeframe and within a separation channel length of only 200 μm. Emrich et al. (2002) performed a high-throughput genotyping experiment using a microfabricated device that comprised 384 CE lanes arrayed radially on a 200-mm-diameter substrate. Each CE lane incorporated an injector and a CE separation channel. On-chip detection was accomplished with a rotary confocal fluorescent scanner. This simultaneous genotyping experiment was accomplished in only 325 sec, and up-to-date represents the highest throughput CE experiment ever achieved. A relevant application of a multiplexed microfluidic device (Fig. 1A) in the proteomics arena, a fast sodium dodecylsulfate (SDS) separation of a protein ladder with laser-induced fluorescence detection, is shown in Figure 1B (Bousse et al., 2001). The microchip device utilized for this separation was approximately 17.5 × 17.5 mm, the separation was performed in 45 sec, and the microchip was capable of sequentially analyzing 11 different samples.

The interfacing of microfluidic devices to mass spectrometry using either ESI or matrix-assisted laser desorption ionization (MALDI) interfaces, while already demonstrated in 1997, continues to be a challenge for a number of reasons: First, the fabrication of high-performance microchip-integrated electrospray emitters can be accomplished only by using specialized microfabrication techniques that work well mainly in silicon or polymeric substrates, but not in glass (Licklider et al., 2000; Schultz et al., 2000; Le Gac, Arscott, & Rolando, 2003); many complex analytical processes, on the other hand, have been demonstrated in glass chips due to the ease of manipulating fluid flows through an electroosmotic mechanism; alternatively, the integration of stand-alone electrospray emitters with distinct microfluidic platforms poses additional challenges, regardless of the material used for chip fabrication. Second, fluid propulsion in CE and CEC is accomplished with the aid of electrical fields, and maintaining stable fluid flows in the direction of electrospray emitters, while continuing the application of electrical voltages to the terminus of these separation elements, requires the development of specialized interfaces that are not easy to integrate within a microfabricated format. Third, the implementation of alternative fluid propulsion mechanisms that generate fluid flows compatible with mass spectrometric detection, such as pressure-driven approaches (Paul, Arnold, & Rakestraw, 1998; Lazar et al., 2000; Lazar & Karger, 2002), electrochemical induced transport (Xie et al., 2004), and piezo (Miliotis et al., 2000), or centrifugal dispensing (Hirschberg et al., 2004), have been investigated only recently, and do not find widespread use yet.

The recent past has witnessed, however, the development of clever solutions that address many of the challenges of microchip-MS interfacing. These accomplishments demonstrate that microfabricated devices have the potential to be a significant advance, relative to traditional sample introduction techniques, for the performance of both ESI and MALDI mass spectrometry. Instrument miniaturization is a rapidly developing field, with the early stages of commercialization already upon us. The overall goal of this review is to provide the reader with an appreciation of the power of microchip devices and their future potential for bioanalysis when coupled with MS. In the following, we present background information on microchip manufacturing, discuss microchip designs that enable interfacing to MS, highlight the benefits and limitations associated with these structures, and demonstrate relevant applications. Future prospects and developments will also be discussed.

II. FABRICATION OF MICROFLUIDIC DEVICES

The fabrication of microfluidic devices involves processes developed earlier for semiconductor manufacturing. Microchip devices, typically a few cm^2, can be fabricated from a variety of materials such as glass (Harrison et al., 1992; Jacobson et al., 1994a,b,c), quartz (Jacobson & Ramsey, 1995a; Jacobson, Moore, & Ramsey, 1995b), silicon (Harrison, Glavina, & Manz, 1993; McEnery et al., 1999), and polymeric substrates (Martynova et al., 1997; Roberts et al., 1997; Ford et al., 1998; Duffy et al., 1999; Xu et al., 2000). The choice of a particular material depends on its surface properties, ease of fabrication, disposability, and price. Unlike devices with on-chip optical detection where high transparency and/or low background fluorescence are essential, the optical properties of the material used for MS coupled microdevices are less critical. Thus, completely opaque materials like glassy carbon can be used as a microfabrication substrate (Ssenyange et al., 2004). Care must be exercised in selecting a material with sufficient chemical stability to prevent the formation of analyte adducts from the microdevice itself. For example, we have observed strong adduct formation with microdevices made of epoxy resins (monomer adducts) and high-alkali content glasses (alkali metal adducts). For practical use, additional important material properties are related to surface activity—adsorption and electroosmosis. Since the surface-to-volume ratio of microfabricated channels is high, sample adsorption on the microdevice channel surfaces must be carefully minimized. Fortunately, in comparison with traditional sample handling protocols that involve sample processing and

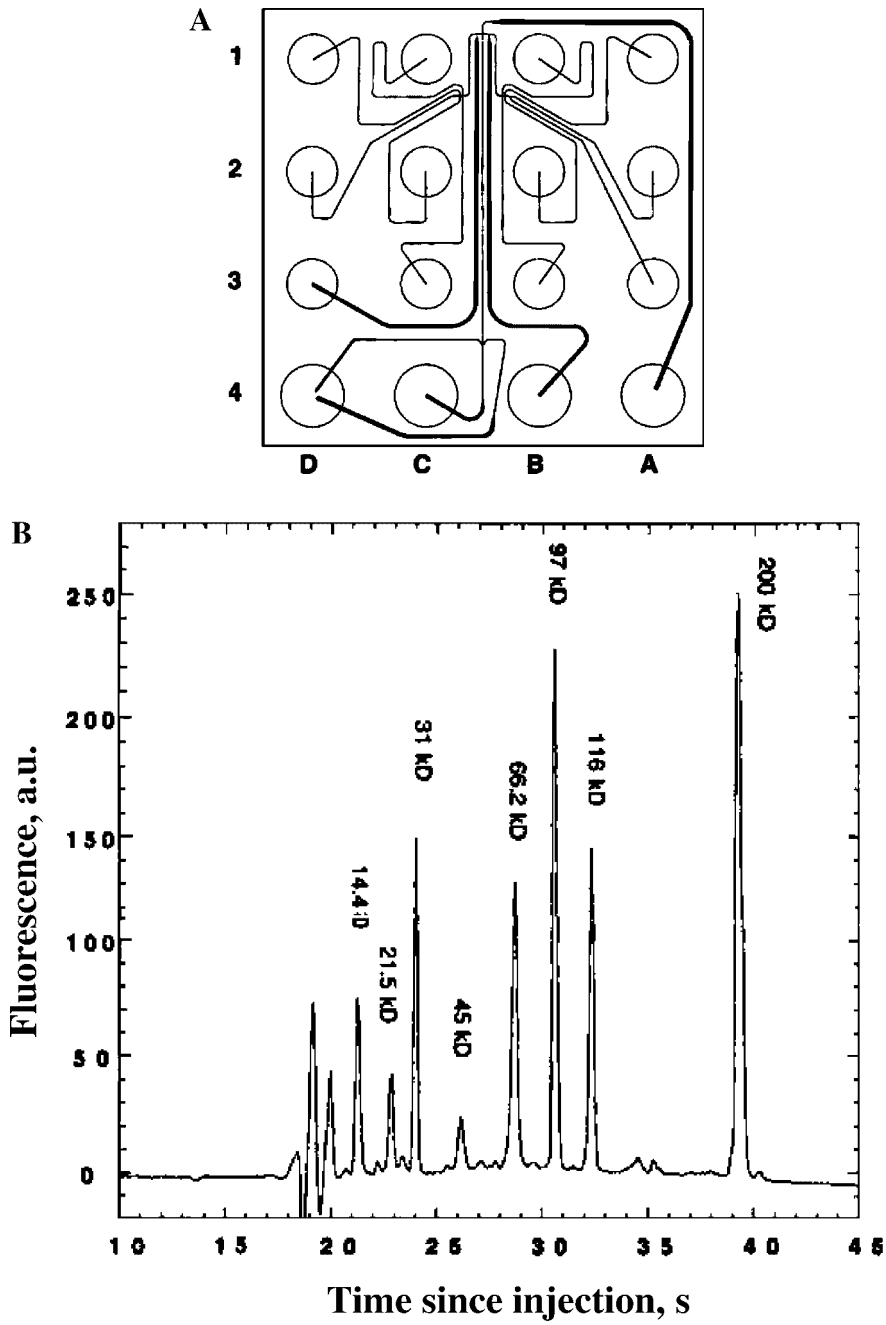

FIGURE 1. Protein sizing on a microfabricated device. **A**: Schematic of an 11 multi-sample microfabricated device that performs protein sizing by integrating on the chip the following steps: noncovalent fluorescent labeling of SDS-denatured proteins, separation, virtual destaining by dilution of the SDS below critical micelle concentration before detection, and detection; wells A4 and C4 are for the separation buffer and waste; B4 and D3 are used as load wells; D4 is the SDS dilution well; all other wells contain sample; (**B**) Electropherogram of a standard protein ladder separation with SDS containing buffer and laser-induced fluorescence detection. Reproduced with permission from Anal. Chem. 2001, 73, 1207–1212. Copyright 2001 American Chemical Society.

transfer among multiple devices, the integration of functional elements on a microchip reduces the total surface exposed to contact with the sample (Figeys & Aebersold, 1999). However, while most work to date does not address the adsorption issue in detail, analyte interaction with surfaces must be minimized for the analysis of low-abundance sample components. Currently, the protocols developed for CE in fused silica capillaries (e.g., low-pH buffers, charged or neutral hydrophilic surface coatings) can be utilized to control adsorption and electroosmosis on microfluidic devices (Weinberger, 1993).

The most frequently used procedure to manufacture microfabricated devices relies on transferring a master pattern onto the surface of a desired substrate by photolithography, followed by removal of the outlined material by wet chemical etching in a liquid phase, or dry etching in a gas-phase plasma. Additional approaches involve metal or resist deposition on given substrates and silicon dioxide or nitride growth on silicon substrates (Madou, 1997). Finally, laser machining, casting, molding, or stamping can be utilized for polymeric materials (Martynova et al., 1997; Roberts et al., 1997; Ford et al., 1998; Duffy et al., 1999; Xu et al., 2000).

The most common photolithography/wet chemical etching technique that uses glass, quartz, or silicon is shown in Figure 2. The microdevice substrate is coated with a fine (100 nm) protective layer of metal (gold or chromium), followed by a thin (0.4–2 μm) and uniform ±(1–3)% layer of photoresist. Next, a photomask with the image of the desired structure is placed in direct contact with the photoresist-coated surface of the wafer and exposed to UV radiation (350–500 nm). The photochemical reaction during exposure will either weaken the polymeric structure of the photoresist (positive resist) or strengthen it (negative resist). During the subsequent chemical development process, the photoresist is removed from either the exposed (positive) or unexposed area (negative). Next, the underlying metal layer is removed, and the exposed substrate is etched (HF or HF/NH_4F solution for glass substrates) until the desired channel depth (1–50 μm) is obtained. Finally, after the removal of protective layers and thorough cleaning, the etched channels are sealed by thermal bonding (e.g., 500–600°C) to a cover plate. The photomask itself is prepared on a glass or quartz plate coated with a UV-absorbing metal layer (gold, chromium, 800–1,000 Å) with the image pattern being created using laser or e-beam technologies (Madou, 1997).

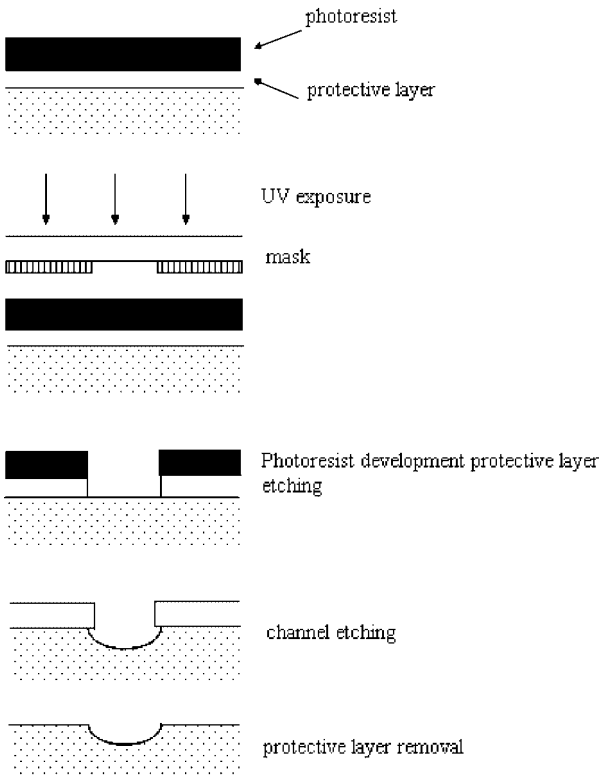

FIGURE 2. Photolithographic microfabrication process with a positive photoresist.

There are several microfabrication parameters that must be considered when choosing the material and method for preparing microdevices, including aspect ratio (i.e., etch depth/width), critical dimension (minimum size feature on the device), linewidth, and resolution. If an anisotropic etch profile that ensures a high-aspect ratio channel is desired, a dry etching technique may be preferred to the above wet chemical etching protocol. Plasma etching and reactive ion etching are the most frequently used techniques for this purpose. Reactive neutral or ionic species (atomic or molecular aggregates) generated in the plasma, adsorb on the substrate and form volatile products with the layer that must be removed. The concentration of the neutral atoms, free radicals and ions, as well as the ion energy, are important factors in controlling the etch rate and the degree of anisotropy (Madou, 1997). As an example, deep reactive ion etching has been utilized in the fabrication of on-chip silicon nozzles for electrospray generation (Schultz et al., 2000; Griss et al., 2002).

Photolithography generally produces satisfactory results with features larger than 1 μm and aspect ratios of less than 10. For the creation of submicron-sized, high-resolution, and high-aspect ratio elements, alternative lithographies such as X-ray, charged beam (e-beam, ion beam), or combined techniques such as LIGA (german acronym for X-ray lithography, electrodeposition, and molding) should be considered. Associated with the given process, new resists and masks (contact or proximity) must be developed; however, their fabrication is still challenging and often expensive. Charged beam lithographies can be used as direct write techniques that make no use of a mask.

In addition to inorganic structures, polymeric materials are attractive substrates for fabricating microchips as they are inexpensive and allow for low-temperature sealing. Polymeric microfabricated devices (polydimethylsiloxane, polyimide, polystyrene, polycarbonate, cellulose acetate, polyethylene terephthalate, etc.) can be prepared by a variety of techniques. The most commonly used procedures include UV-laser photoablation (Roberts et al., 1997), casting of a polymer against a master with the imprinted microchannel network (Duffy et al., 1999), hot wire/silicon template imprinting (Martynova et al., 1997), pressure imprinting using a silicon template (Xu et al., 2000), and X-ray lithography (Ford et al., 1998; Meng et al., 2001). Recently, a slot type planar electrospray tip fabrication process using photolithography in SU-8 epoxy resin has been described. This approach provides a potential for simple creation of separation columns with integrated electrospray tips (Le Gac, Arscott, & Rolando, 2003). It is beyond the scope of this article to provide a complete review of the microfabrication processes. Microfabrication methods used for microfluidic chip applications are reviewed by Becker & Gärtner (2000) and Ziaie et al. (2004); additional useful information can be found on the following web pages: National Nanofabrication Users Network (http://www.nnin.org) and Microelectronic Center Denmark: (http://www.mic.dtu.dk/Research/BCMS/MicroTAS.aspx/microtas.htm).

III. BASIC DESIGNS OF MICROCHIPS INTERFACED TO MASS SPECTROMETRY

A. Functional Elements and Microfluidic Manipulations

The microfabrication of functional elements that perform specific tasks and their integration in microscale systems capable of performing complex operations are described in numerous articles (Jakeway, de Mello, & Russell, 2000; Auroux et al., 2002; Reyes et al., 2002). Four basic operational elements must be implemented on a microchip to ensure the functionality of a separation microchip-MS device: (1) separation element; (2) sample injector; (3) microfluidic propulsion element; and (4) microchip-MS interface. The development of separation processes on microchip devices takes full advantage of the integration capabilities of microfabrication. Transfer lines, distribution, injection, and detection elements can be fabricated on a single substrate, and thus the inherent problems associated with the use of unions and transfer capillaries (complexity, dead volume, limited multiplexing capabilities) are minimized.

As an example, a microfluidic device for performing 2D-liquid phase separations has been developed and applied for the separation of peptide samples from tryptic digests of standard proteins (Rocklin, Ramsey, & Ramsey, 2000). Total analysis time was less than 10 min, with the second dimension being completed in a few seconds, and the peak capacity of the 2D system was estimated to be in the 500–1,000 range. In order to identify individual analytical species, the interfacing of microfluidic devices to MS detection is essential. Time-of-flight (TOF)-MS detection with its rapid, non-scanning characteristics represents an ideal match for the high speed potential of microchips; however, other MS instrumentation can also be successfully employed.

1. Separation Element

While CE and CEC separations are most frequently performed on microfluidic devices, some efforts are now focused on implementing μLC as well. The demonstrated power of LC for the separation of peptide mixtures and increased loading capacity makes this separation approach an important addition to microchips. An obvious strategy is to load chromatographic packing in the channels of the microdevice (Colon, Maloney, & Fermier, 2000; Jemere, Oleschuk, & Harrison, 2003). While potentially effective, more work is needed to demonstrate the robustness and performance of such approaches. Currently, polymeric monolithic columns, in which a porous structure is polymerized in the column, are being developed, especially for capillary column dimensions ($\leq$ 75 μm i.d.). These columns are characterized with high efficiencies (10^5 plates/meter or greater) and high permeability resulting in low pressure drop (Gusev, Huang, & Horvath, 1999). Since such approaches do not require packing of the channels, the synthesis of polymeric monolithic stationary phases in microdevices is being currently explored (Svec, 2004). These materials may offer significant potential, especially for multichannel designs. For example, Peterson et al. (2003) has prepared a dual-function capillary microanalytical device by *in situ* photolithographic grafting of a porous polymer monolith that performed on-line solid-phase extraction and enzymatic digestion for peptide mass mapping. Fintschenko et al. (2001) has demonstrated the separation and detection (with laser-induced fluorescence) of polycyclic aromatic hydrocarbons on a microfluidic CEC chip that comprised an acrylate-based, UV-patterned porous polymer monolith; Throckmorton, Shepodd, & Singh (2002) has performed on a similar chip the separation of bioactive peptides in less than 45 sec, demonstrating up to 600,000 plates/m separation efficiency. Alternatively, Lazar et al. (2003) has interfaced to ESI-TOFMS a glass microfluidic chip that integrated a UV-patterned monolithic CEC separation system that was used for the separation of low fmol amounts of standard protein digests. The use of polymeric monolithic packings for chip electrochromatography was recently reviewed by Stachowiak, Svec, & Fréchet (2004). Simple fabrication/patterning ability through photoinitiated polymerization, amenability to high-throughput manufacturing, and low hydraulic resistance make these monolithic media very attractive choices for the production of LC separation architectures with distinct functionality. Finally, a third approach involves microfabricating the chromatographic packing within the microdevice itself. Using deep reactive ion etching in quartz, $5 \times 5 \times 10$ μm microfabricated support elements, so-called collocated monolith support structures (COMOSS), separated by 1.5 μm wide and 10 μm deep rectangular channels, have been constructed (He, Tait, & Regnier, 1998). Alternatively, COMOSS chips were fabricated in PDMS as well (Slentz, Penner, & Regnier, 2002). After chemical bonding of a polymeric stationary phase to the surface of the monolith structure, successful reverse-phase chromatography has been achieved. In summary, it is clear that the integration of LC systems on microfluidic platforms is a very active field, and LC/MS will no doubt be an important future technology with microfabricated components.

2. Sample Injection

Sample injection in CE, CEC, or μLC separation channels can be accomplished using electrical fields through a cross or double-T injector configuration (Harrison et al., 1992; Jacobson et al., 1994a). If channel dimensions are properly chosen, pressure or vacuum injections can also be performed (Zhang, Foret, & Karger, 2000). On-chip integration of microreactors, for example microdigestion chambers (Jin et al., 2003; Peterson et al., 2003), may enable on-line sample processing followed by the separation of the resulting products and detection.

3. Microfluidic Propulsion Element

An important feature regarding microchip operation is the approach utilized to drive the fluids and reagents through the microchip channels. Microfluidic manipulations for analytical purposes have been mainly performed by means of electrical forces (Harrison et al., 1992; Harrison, Glavina, & Manz, 1993; Jacobson et al., 1994a,b,c) or pressure gradients (Zhang et al., 1999; Zhang, Foret, & Karger, 2000). If electrical forces are used, fluid flow-streams (electroosmotic flows) follow the direction of the potential gradient, and electrokinetic fluid mixing or splitting

can be accomplished by the applied potentials. This approach has been employed with on-chip detection, typically by means of laser-induced fluorescence. However, stable operation of microchip systems interfaced off-chip with mass spectrometry detection frequently requires additional pumping to generate or redirect fluid flows towards an electrospray tip. This is typically accomplished by using either a pressure gradient (Xue et al., 1997a; Zhang et al., 1999) or external electroosmotic pumping (Figeys, Ning, & Aebersold, 1997; Figeys & Aebersold, 1998a; Figeys et al., 1998b,c). Alternatively, in the case of microchips equipped with fine nanospray emitters, fluid flows (20–30 nL/min) are generated by the electrospray process itself (Lazar et al., 1999). On-chip multichannel electroosmotic pumping systems have been constructed and used in conjunction with ESI interfaces for sample infusion (Lazar & Karger, 2002). Pressures in excess of 8,000 psi were generated by electrokinetic pumping in packed capillaries (Paul, Arnold, & Rakestraw, 1998), and the transfer of this technology to microfabricated platforms is anticipated. Recently, an on-chip electrochemical pumping system based on electrolysis was fabricated on both silicon and glass substrates. The gas formed by electrolysis allowed pumping at backpressures as high as 200 psi at 20 nL/min flow rate. Solvent gradient was generated using two electrochemical pumps and confirmed by MS monitoring with a polymer electrospray nozzle (Xie et al., 2004). In addition, pumping systems based on piezoelectric actuation (Laurell, Wallman, & Nilsson, 1999; Laurell, Nilsson, & Marko-Varga, 2001) have also been tested for interfacing microchips to MALDI-MS.

B. Microdevice Interfaces for ESI-MS

Fluid flows in microfabricated devices are of the order of 0–300 nL/min. These values are a close match to the flow rates necessary to operate micro/nano ESI sources (Mann & Wilm, 1994). To date, electrospray from microfabricated devices has been generated directly from the chip surface (Ramsey & Ramsey, 1997; Xue et al., 1997a,b) or using well-known nano/microspray, liquid sheath, and liquid junction interfaces (Figeys, Ning, & Aebersold, 1997; Xu et al., 1998; Figeys & Aebersold, 1998a; Figeys et al., 1998b,c; Bings et al., 1999; Lazar et al., 1999, 2000; Li et al., 1999, 2000a,b; Xiang et al., 1999; Zhang et al., 1999; Zhang, Foret, & Karger, 2000; Meng et al., 2001). More recently, microfabricated emitters integrated in the microchip body have been introduced (Licklider et al., 2000; Schultz et al., 2000; Wen et al., 2000; Le Gac, Arscott, & Rolando, 2003; Arscott, Legac, & Rolando, 2005).

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, to obtain high sensitivities. 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, that is, suppress eluent/analyte flows in the direction of the potential gradient towards a terminal on-chip electrode, such as the case of CE and CEC separations integrated on the chip.

1. *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 (Ramsey & Ramsey, 1997; Xue et al., 1997a,b). Strong electrospray signals were generated by infusing peptide and protein samples through the chip either by a syringe pump (Xue et al., 1997a) or by electroosmosis (Ramsey & Ramsey, 1997). To prevent spreading of the liquid when exiting the microchip channel, the chip surface was hydrophobically silanized around the channel exit (Xue et al., 1997a). This design was incorporated into a multiple-channel microdevice interfaced to ESI-MS for the analysis of protein digests (Xue et al., 1997b). Recently, the open channel electrospray properties were studied in more detail for devices made of a dielectric, nonwetting material (Lozano, Martinez-Sanchez, & Lopez-Urdiales, 2004). In accord with previous experimental results (Huikko et al., 2003), it was concluded that the electrospray activated from a small opening on a flat hydrophobic poly(dimethylsiloxane) surface can have performance close to that of a needle arrangement (Svedberg et al., 2004).

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 approximately 12 nL (Ramsey & Ramsey, 1997). For microchip separations where peak volumes are typically below ~5 nL, any separation would thus be lost in the dead volume of the electrospray cone. As with column separations, a sharp electrospray tip is required to minimize dead volumes and to improve ionization efficiency.

2. *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 (Bings et al., 1999; Lazar et al., 1999; Zhang, Foret, & Karger, 2000) or a fused silica capillary transfer line was inserted in the microchip body (Figeys, Ning, & Aebersold, 1997; Figeys & Aebersold, 1998a; Figeys et al., 1998b,c). For example, an aminopropyl silane derivatized capillary that acted as an external electroosmotic pump was inserted in the channel, perpendicular to the surface of the chip, to transfer (draw) sample from the microchip channel to the microelectrospray interface (Fig. 3A). Microspray tips can also be inserted from the chip edge as shown in Figure 3B (Li et al., 2000b). An electrospray stabilizing buffer solution was infused with the aid of a syringe pump from a side channel placed close to the chip terminus, and the electrospray voltage was applied either to the chip or to a gold-coated microspray tip. Alternatively, the spraying capillary was inserted in a liquid sheath interface (Li et al., 1999).

Efforts have been made to achieve stand-alone operation of the microchip-ESI interface, that is, without external devices connected to the chip. One approach involved the use of a

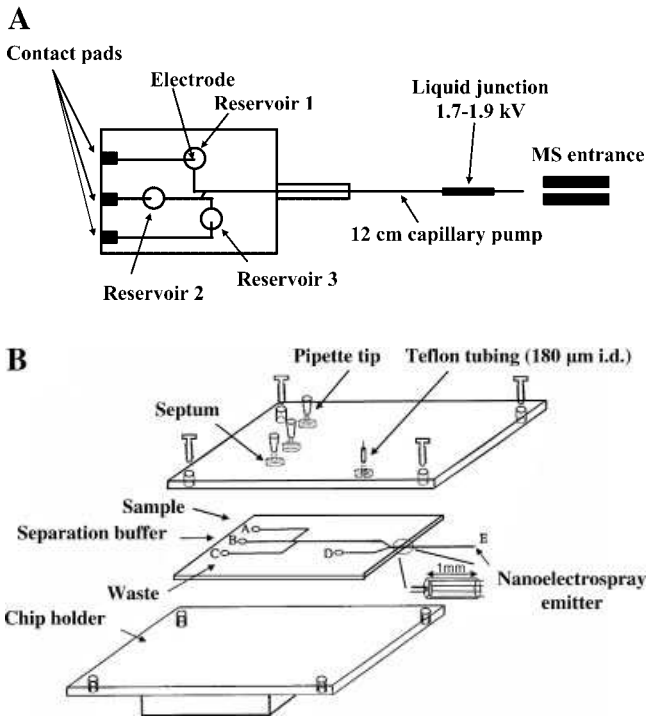

FIGURE 3. Microchip configurations with inserted ESI needles. **A**: Schematic representation of the microchip configuration for MS infusion with an inserted capillary that acts as an electroosmotic pump and transfer line between the chip and ESI interface. Reprinted with permission from Figeys et al. (1997); (**B**) Schematic representation of the microchip configuration for CE separation and MS analysis using a capillary ESI emitter. Reproduced with permission from Anal. Chem. 2000, 72, 599–609. Copyright 2000 American Chemical Society.

nanospray emitter (5 μm i.d.) inserted in the microchip channel (Lazar et al., 1999). External assistance for stabilizing the spray was not required since fluid flow necessary to sustain the signal was generated by the electrospray process itself. Full mass spectra were acquired with a TOFMS at 50–100 spectra/sec storage rates (10–20 msec/spectrum), and the detection of 340–500 zeptomole amounts of peptides and proteins from 100 nM solutions was achieved. If needed, an auxiliary side-channel can be connected to the main channel via an electrically permeable glass membrane to generate the EOF flow towards the electrospray exit port (Lazar et al., 2000). In this case, the microchannel itself can act as an electroosmotic pump that delivers the eluent to the electrospray tip. Alternatively, the microfluidic side-channel can be modified with a neutral coating (e.g., polyacrylamide) to remove the EOF (Ramsey & Ramsey, 1997).

Using an alternative microchip-ESI source design, a liquid junction configuration with a removable electrospray tip inserted in a subatmospheric electrospray interface (Foret et al., 2000), has been developed, see Figure 4 (Zhang et al., 1999; Zhang, Foret, & Karger, 2000). Proper adjustment of the pressure in the interface ensured stable sample delivery to the spray and mixing of the CE buffer with a spray solution (acidified aqueous methanolic solution) from the liquid junction. In the first design, the liquid junction was external to the chip; however, it can be also integrated in the microfabricated device itself (Zhang, Foret, & Karger, 2000). A free-standing liquid junction interface suitable for coupling ESI to the flat edge of a CE microchip was also reported (Wachs & Henion, 2001; Deng, Zhang, & Henion, 2001a; Deng et al., 2001b). In this case, a suitable solvent that is

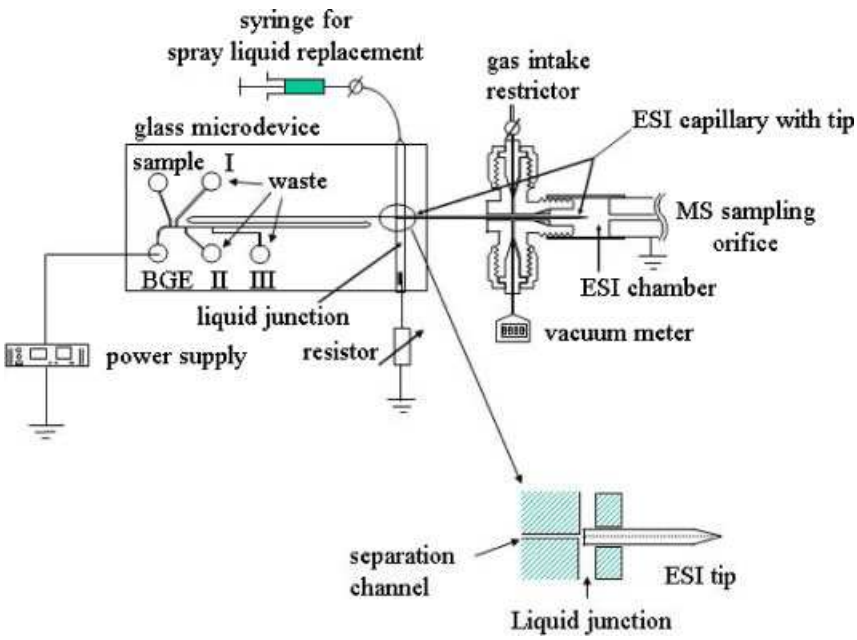

FIGURE 4. Microchip device with an integrated liquid junction interface for high performance CE-MS analysis. See Figure 10 for results using this device. Reproduced with permission from Anal. Chem. 2000, 72, 1015–1022. Copyright 2000 American Chemical Society. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

continuously delivered with the aid of an external pump (μL/min) to the liquid junction interface collects and ensures proper transport of the microchip CE effluent and sample through the pneumatically assisted electrospray needle.

Precise, low dead volume alignment of the ESI tip with the separation channel (typically less than 100 μm) is essential for maintaining separation efficiency in these microchip designs. Guiding channels prepared by double etching in the chip substrate help in producing a fixed, rigid structure with precise alignment between the channel and capillary (Zhang et al., 1999; Zhang, Foret, & Karger, 2000). A close match between the optimum separation and electrospray flows is also important in maintaining stable electrospray signal and minimizing band broadening. An auxiliary channel connected to the exit of the separation channel, or alternatively a liquid junction, has been used to decouple the separation from the ESI and improve the overall performance of the system (Zhang, Foret, & Karger, 2000).

3. Spray Generation from Microchip Integrated Electrospray Emitters

Batch-generation of microchips with integrated electrospray emitters/tips can result in improved emitter reproducibility and the potential of 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 5×10 μm rectangular openings that extended 1 mm beyond the edge of the substrate) were fabricated from parylene polymer layers deposited on a silicon substrate (Licklider et al., 2000). Microfabricated electrospray nozzles with high aspect ratio (10 μm i.d. $\times$ 50 μm depth) were constructed on the planar surface of a silicon substrate using deep reactive ion etching (Schultz et al., 2000), see Figure 5. The latter procedure allowed for the creation of a large number of ESI nozzles in parallel, with

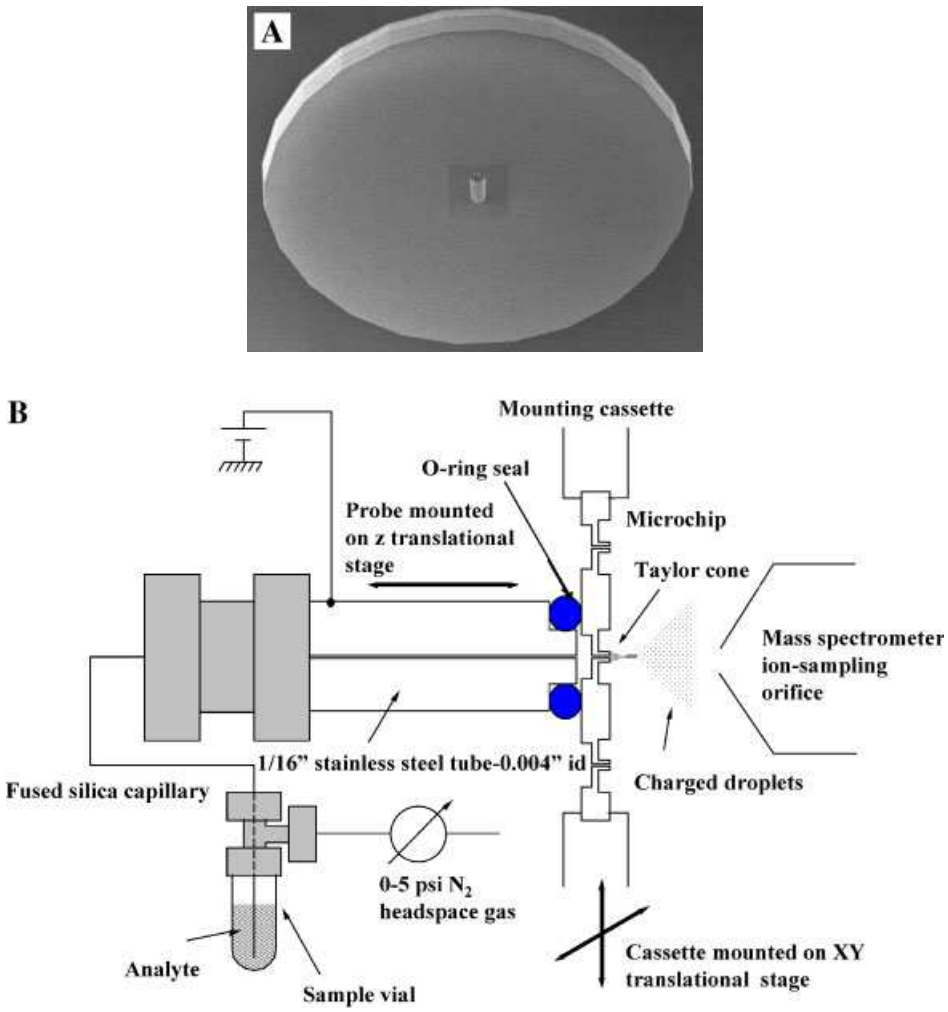

FIGURE 5. Microchip with integrated electrospray emitter. **A**: SEM image of a microfabricated silicon electrospray nozzle; (**B**) Schematic representation of a complete chip handling and fluid delivery system. Reproduced with permission from Anal. Chem. 2000, 72, 4058–4063. Copyright 2000 American Chemical Society. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

similar performance to that of microspray capillaries. Stable electrospray was demonstrated by infusing dilute standard solutions (100 nM) of proteins through pressurized tubing connected to the back of the silicon substrate. During operation, the silicon wafer with multiple electrospray tips was mounted in a cassette on an XY translational stage. These silicon ESI Chips™ in a 400 nozzle format are presently commercialized devices (www. advion.com). Using a similar approach, Griss et al. (2002) and Sjödahl et al. (2003) used batch silicon microfabrication and oxidation for the fabrication of an array of tapered SiO_2 electrospray needles. 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 (Arscott, Legac, & Rolando, 2005).

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 example, a microfluidic chip was fabricated by embossing in Zeonor 1020R polymer, and this chip incorporated between the substrate and the cover plate a 5-μm-thick film of parylene C. For electrospray generation, the parylene film was micromachined in a triangular shape by lithography and etching (Kameoka et al., 2002). In another approach, the microfluidic system and the electrospray exit nozzle were fabricated by plasma etching in polyimide (Gobry et al., 2002; Lion et al., 2004a). The starting material, copper-coated polyimide (industrially used for the production of flexible printed circuits) was covered with photoresist, exposed to light, developed, and the protected copper was chemically etched to leave exposed polyimide for microchannel fabrication. Following several steps of chemical and plasma etching that enabled the fabrication of microchannels, access holes and integrated gold-coated electrodes, the microchannels were enclosed with laminated polyethylene terephthalate foil and cut on the edge to produce a tip for electrospray generation. A similar approach has been employed for the commercial fabrication of microfluidic chips that integrated HPLC channels with enrichment modules and ESI emitters; these systems were demonstrated for the subfemtomole detection of protein digests (Yin et al., 2005, www.agilent.com). In addition, electrospray emitters were fabricated from SU-8 epoxy resin (Fig. 6) by photolithography and dicing (Le Gac, Arscott, & Rolando, 2003), from polyethylene terephthalate (Rohner, Rossier, & Girault, 2001) and polycarbonate substrates (Tang et al., 2001) by laser ablation, from poly(dimethylsiloxane) by casting (Kim & Knapp, 2001; Chiou et al., 2002; Huikko et al., 2003; Svedberg et al., 2004), and from poly(methylmethacrylate) by injection molding (Svedberg et al., 2003), atmospheric molding (Muck & Svatos, 2004), micromilling (Schilling et al., 2004), and mechanical cutting (Yuan & Shiea, 2001). The performance of these microfabricated emitters was most often tested with peptide/protein solutions (1–10 μM), and good quality mass spectra were typically generated at the 1 μM concentration level.

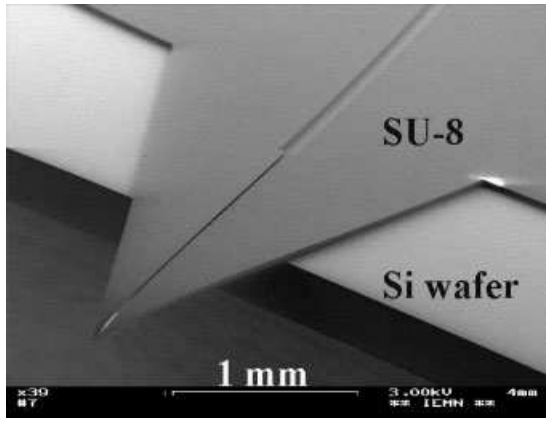

FIGURE 6. SEM image of a planar ESI emitter fabricated from negative SU-8 photoresist. Reproduced with permission from Electrophoresis 2003, 24, 3640–3647. Copyright 2003 Wiley InterScience.

C. Microdevice Interfaces for MALDI-MS

Matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) is another major MS technology for the analysis of proteins and peptides. Unlike electrospray, MALDI is typically performed inside the vacuum source of the mass spectrometer on a solid spot of sample mixed with a suitable matrix for ionization. Since multiple sample spots can be deposited on the same target, MALDI-TOF-MS can, in principle, provide much faster sample processing times than ESI-TOF. Consequently, the development of microanalytical platforms with MALDI-MS detection that can accommodate high-density sample processing elements will benefit applications that require high-throughput analysis.

The potential for on-line MALDI analysis of multiple sample streams, generated by separation systems and deposited in parallel on a moving tape, has been demonstrated (Preisler, Foret, & Karger, 1998; Preisler et al., 2000). The interfacing of chip-based separations with MALDI, in a single or parallel format, could be performed either on-line or off-line using similar interfaces. A microfabricated piezo-actuated flow-through dispenser was utilized to deposit samples on standard or micromachined silicon nanovial target plates (nanovials size was 100×100 μm to 400×400 μm and 20 μm deep) for MALDI-MS analysis (Öuml;nnerfjord et al., 1998; Ekström et al., 2000; 2001). Droplet size varied from 30 to 200 pL, the maximum ejection frequency was 500 Hz, and this sample preparation technique enabled the detection of low amol/fmol amounts of protein. A similar flow-through microdispenser with an internal volume of 250 nL was used to deposit discrete droplets and to interface on-line capillary liquid chromatography to MALDI-TOF-MS (Miliotis et al., 2000). Miniaturization has proven here again its benefits, since the capability of performing repetitive microdispensing and accurate deposition of 60-pL droplets of a dilute sample, to a confined area of 300×300 μm allowed for on-spot sample enrichment and 10–50 times signal amplification. On-chip CE-MALDI was accomplished by performing the separation in the matrix solution in an open CE channel, evaporating the solvent, and scanning the CE channel with the desorption laser beam (Liu et al., 2001). Capillary isoelectric focusing (CIEF)-MALDI was demonstrated in poly(methyl-methacrylate) chips with imprinted pseudo-closed channels that were covered only during the IEF step. The matrix solution was added after protein focusing, and MS detection occurred in the open channel (Mok et al., 2004).

A high-density fully automated compact disc (CD) format microfluidic system has been developed for sample preparation prior to MALDI-MS analysis (Gustafsson et al., 2004; Hirschberg et al., 2004). This system is now commercially available (www.gyros.com), and although not used for on-line separations coupled to MALDI, the CD format provides sufficient space for fabrication of several separation columns suitable for sample fractionation (Spesny & Foret, 2003). The samples and solvents that are to be processed in parallel are loaded in miniature reservoirs near the center of the disc, the fluids are pumped through microchannels and microchambers towards the outer edge of the CD by centrifugal force, and the samples are collected in small spots (200 × 400 μm) at the end of each working line on the CD. MALDI-MS on the collected spots was thus performed in a high-throughput format on a CD comprising 96 sample processing lines. Each line comprised numerous steps: sample preconcentration, affinity selection, desalting, and digestion. The capability to handle sample volumes as low as 1 μL, and to detect sample amounts as low as 500 amol–5 fmol, was demonstrated with these centrifugal devices (Fig. 7). Using another approach, a continuous flow glass/silicon microchip that integrated a microdigestion reactor was physically incorporated into the standard MALDI sample plate of an MS instrument (Brivio et al., 2002). The vacuum of the MALDI-MS vacuum chamber was used as a driving force for inducing flows in the reaction microchannels. The device was demonstrated for the analysis of nucleotides and peptides.

A separate field where miniaturization meets biology oriented applications is represented by a technique frequently referred to as protein chips. Typically, these systems are based on arrays of immobilized proteins (antibodies or affinity targets) and optical evaluation of the protein–ligand binding. The use of chips for surface plasmon resonance may serve as an example (Gilligan, Schuck, & Yergey, 2002; Borch & Roepstorff, 2004; Grote et al., 2005). However, these chips can also be coupled to MALDI mass spectrometry to confirm the identity of the analytes. Borrebaeck et al. (2001) has fabricated silicon chip nanovial MALDI target plates coated with recombinant scFv antibody fragments that were used for the analysis of specific antibody–antigen interactions between scFv and choleratoxin. Surface-enhanced laser desorption ionization (SELDI) target plates prepared as chips for selective capture of analytes are marketed as protein chip arrays (www.ciphergen.com). Since this technology only touches the scope of this article, more information can be found in a recent review (Tang, Tornatore, & Weinberger, 2004). Alternatively, microfabrication techniques have been used to fabricate enzyme-functionalized MALDI plates for the structural analysis of ribonucleic acids (Berhane & Limbach, 2003), and to prepare MALDI sample targets with twin anchors for improved mass accuracy (Sjödahl et al., 2005). Microfluidic approaches for moving fluids based on electrowetting forces were utilized for in-line sample purification prior to MALDI-MS detection (Wheeler et al., 2005). In summary, although more research is needed to develop an efficient interface between separation microdevices and MALDI-TOF, the combination of the two techniques should be straightforward since the features developed for sample preparation on microchip structures for ESI-MS can be directly transferred onto microchips for MALDI-MS.

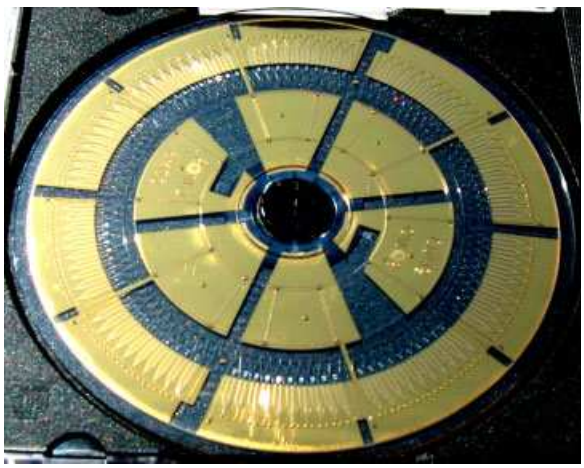

FIGURE 7. Photograph of a centrifugal CD for high-throughput MALDI-MS from a chip. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

D. Applications

1. Sample Infusion Analysis

Although the development of microdevices interfaced with mass spectrometry is recent, a number of articles describe promising results in design, development, and practice. Early microfluidic devices were used mainly as sample delivery vehicles for infusion experiments, with the potential to load rapidly a large number of different samples. Originally, individual channels were used for each sample to prevent cross contamination (Xue et al., 1997a,b). The difficulty in spraying from the flat surface of the chip led to designs with an array of channels connected to an array of capillary electrospray tips, see Figure 8 (Liu et al., 2000), or with multiple sample entries connected to one ESI capillary inserted in the chip (Figeys et al., 1998b).

In the first example, the size of the device was selected to be compatible with the standard microtitter well plate. Since a separate ESI capillary was employed for each analysis, concerns related to sample cross-contamination were eliminated. An electro-pneumatic distributor was sequentially used to activate the high voltage for electrospray and nitrogen gas for fluid control. This microdevice was tested with an ion trap MS; however, if fast sample screening is desired, interfacing of such a multichannel device to a high-speed TOF-MS detector can increase throughput even more. In the second design, with a single ESI capillary (where the microchip acted as a sample introduction device and the capillary acted as an electroosmotic pump that aided transfer of the sample to the ESI interface), the sample flow was manipulated by an array of computer-controlled high-voltage relays. Although the potential of sample cross contamination remains an issue, this simple arrangement was successfully used for the analysis of yeast protein digests excised from a 2D gel (Figeys et al., 1998b). Low fmol per microliter

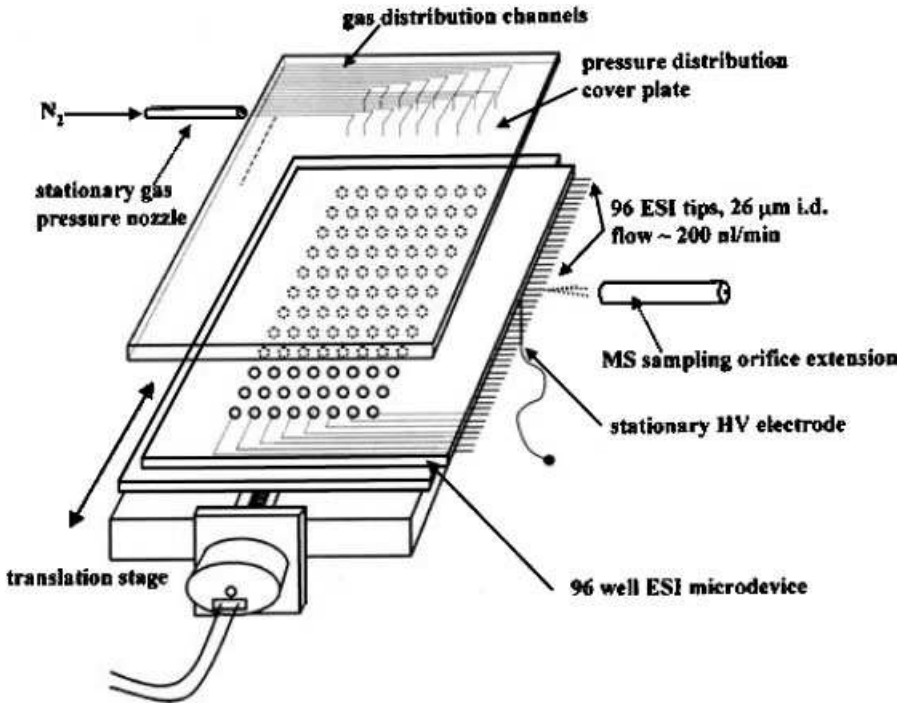

FIGURE 8. Diagram of a multichannel electro-pneumatic sample delivery system with multiple ESI emitters. See text for more details. Reproduced with permission from Anal. Chem. 2000, 72, 3303–3310. Copyright 2000 American Chemical Society.

detection limits and the possibility for automated sequential analysis of multiple samples was demonstrated for tryptic digests produced from 1D and 2D gel electrophoresis of yeast total cell lysates. A modified device was later used to create a solvent gradient for the elution of peptides trapped in a short bed of C18 material packed into the transfer capillary (Figeys & Aebersold, 1998a). While the analytical results were comparable with those obtained with dedicated bench-top equipment, the miniaturization and integration of individual instrument elements facilitated rapid processing of samples available in a very small amount. The fast completion of an analysis is critical when large numbers of samples must be investigated.

Currently, the most widespread mode of infusion analysis utilizes an array of 400 tips microfabricated in silicon. A fully automated instrument employing disposable tip arrays is now commercially available (www.advion.com). In conjunction with pipette tip-based sample desalting (ZipTip®) the system can perform rapid infusion MS analyses without the danger of sample cross-contamination. A number of applications have been demonstrated with this device for the quantitative analysis of drugs in plasma (Kapron et al., 2003; Leuthold et al., 2004), for non-covalent protein–protein and protein–ligand interaction screening (Keetch et al., 2003) and for carbohydrate analysis (Zamfir et al., 2004; Zhang & Chelius, 2004). The capability to perform fully automated mass spectrometric characterization of complex carbohydrate systems (Fig. 9), using a nanoscale liquid delivery system and the chip-based nanoelectrospray tip array assembly, was reported by Zamfir et al. (2004).

2. *Sample Preconcentration*

To improve sensitivity, either on-chip focusing can be performed, for example using transient isotachophoresis (Zhang et al., 1999) or sample stacking, or an external sample preconcentrator can be coupled to the microdevice (Li et al., 2000a). A microchip CE-ESI/Qq-TOF-MS analysis system was used for the fast analysis (1.5 min) of tryptic peptides obtained from a band excised from a 2D gel separation of a membrane protein extract from *H. influenzae* Rd^- strain. Sample volumes of 3 μL were enriched using a C18 membrane solid-phase microextraction (SPME) preconcentrator connected to the microdevice. Sample concentration detection limits of typically 2.5 nM for hydrophobic peptides were demonstrated. Alternatively, peptide samples, preconcentrated on a small C18 cartridge placed between the microchip infusion device and the ESI interface, were analyzed from 0.1 nM solutions (Figeys & Aebersold, 1998a).

More recently, micro-preconcentrators were incorporated within the microfluidic device itself. A silicon microextraction chip that contained reversed-phase packing material immobilized within the chip by a high-aspect ratio vertical grid structure, was used for the preconcentration of 2 nM peptide solutions that were further analyzed by MALDI-MS. The performance of this structure was modeled with the aid of computational fluid dynamic tools (Bergkvist et al., 2002). In another approach, sample clean-up and trace enrichment of peptides from 10 nM solutions was accomplished on microchip structures that incorporated a trap of reversed-phase POROS™

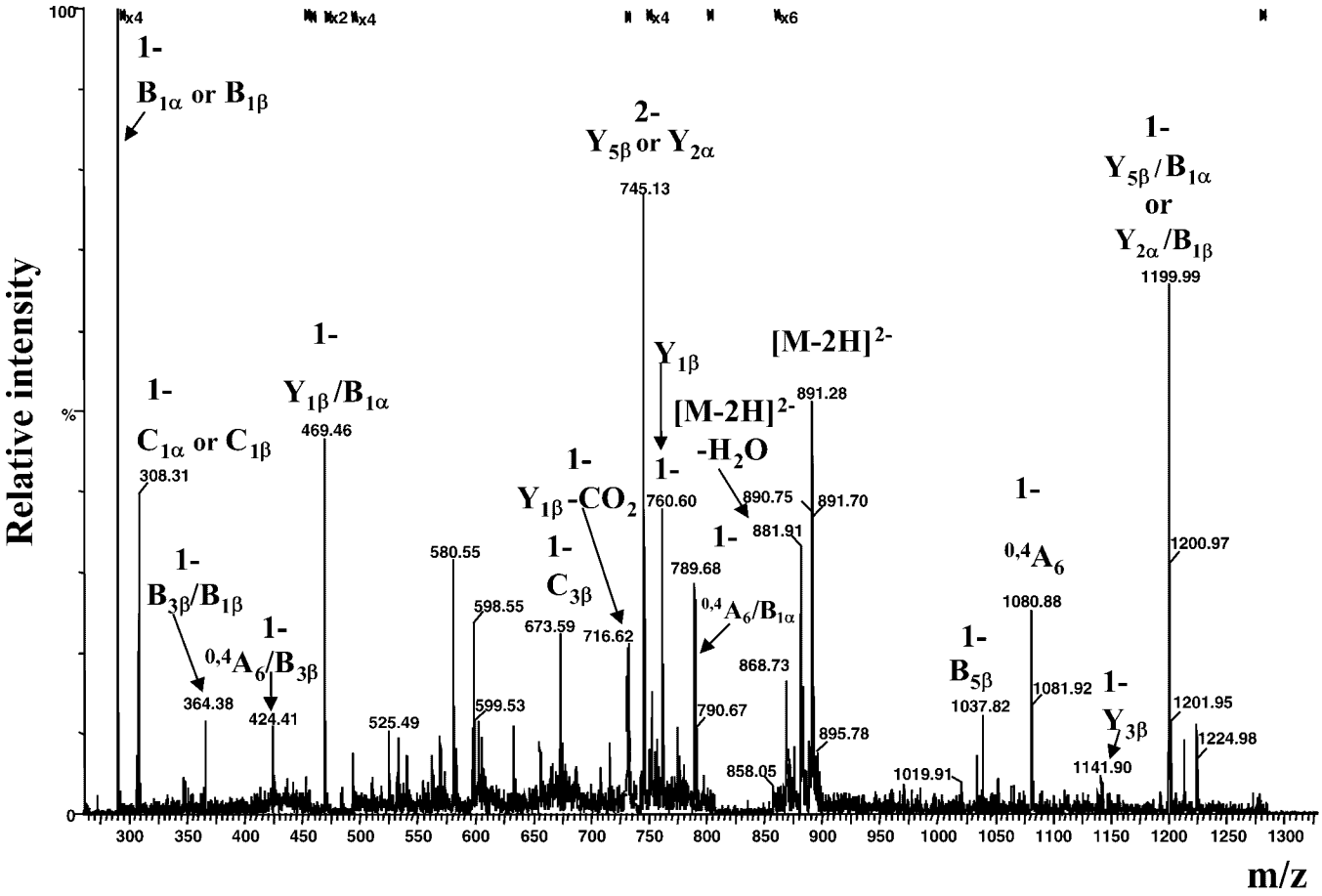

FIGURE 9. Fragmentation spectrum of $NeuAc_2Gal_3GlcNAc_2GalNac$-Ser (m/z 890.32) obtained by automated chip-based (−) nanoESI-QTof-MS/MS infusion analysis. Reproduced with permission from Anal. Chem. 2004, 76, 2046–2054. Copyright 2004 American Chemical Society.

R2 beads; sample transfer to a standard MALDI target plate was performed with the aid of a syringe pump (Ekström et al., 2002). Alternatively, monolithic porous polymeric structures were incorporated into a chip to perform SPME, and a 10^3-fold preconcentration of peptides and proteins was achieved (Yu et al., 2001); or, an array of eight porous methacrylate-based monolithic columns were fabricated in a Zeonor polymeric chip, and demonstrated for the SPE of drugs spiked at a level of 0.025 μg/mL in human urine (Tan, Benetton, & Henion, 2003).

In addition to the use of nonspecific hydrophobic interactions to capture the desired sample components, approaches based on affinity selection have also been tested. For example, peptide preconcentration or affinity capture followed by CE-MS analysis was performed with microfabricated structures packed with C18 particles or c-*myc*-specific antibodies immobilized on protein G-Sepharose beads. These microfluidic chips were used for the isolation of trace-level antigenic peptides spiked at a level of 20 ng/mL in human plasma (Li et al., 2002). Immobilized metal affinity chromatography beads (IMAC) were used for the capture of tryptic phosphopeptides using similar devices or a microfluidic centrifugal compact disk (Hirschberg et al., 2004). The CD format enabled parallel processing of 96 samples simultaneously. In another approach, polymeric methacrylate-based porous monoliths were derivatized with Cibacron-blue-3G-A and used for the affinity capture of albumin from human cerebrospinal fluid (Li & Lee, 2004). Alternatively, PDMS microchips containing poly(vinylidene fluoride) membranes were used for continuous affinity capture and concentration of two model systems including aflatoxin B1 antibody/aflatoxins and phenobarbital antibody/barbiturates (Jiang et al., 2001).

3. *Sample Clean-Up*

Microfabricated devices for sample clean-up/desalting were fabricated by using an excimer laser direct-write system for imprinting microchannels in polycarbonate chips, and by sandwiching a microdialysis membrane between two overlapping substrates (Xu et al., 1998). A dual microdialysis device for the removal of low- and high-molecular weight sample contaminants was coupled on-line to a quadrupole ion trap ESI/MS system (Xiang et al., 1999). In this case, the

polycarbonate block with a serpentine channel was sandwiched between two dialysis membranes. By selecting the molecular weight cut-off of the membranes, the unwanted sample components could be eliminated from the analysis. Efficient sample clean up for DNA and protein samples, with much improved signal-to-noise ratios, were demonstrated. A similar membrane design was also integrated in polyimide-based microdevices for desalting protein samples (Lion et al., 2002, 2003). Alternatively, polymeric monolithic columns prepared in SU-8 microfluidic chips were used for peptide sample desalting and on-line MS analysis (Le Gac et al., 2004).

4. *Protein Digestion in Microfluidic Enzyme Reactors*

A microchip nanoelectrospray device, and a fast liquid phase digestion protocol involving high enzyme:substrate ratios, was utilized for the rapid on-chip digestion (5–15 min) and ESI-TOFMS analysis of proteolytic digestion fragments of hemoglobin variants (Lazar, Ramsey, & Ramsey, 2001). Proteins were identified using peptide mass fingerprinting by loading 0.1–2 μM sample solutions on the chip, with the sequence coverage for the analyzed proteins being in the 70–97% range. An on-line nanoflow sample processing device that included a microchip immobilized enzyme reactor (μ-chip IMER) and a microfabricated piezo-actuated flow-through dispenser, to transfer and deposit the samples from the μ-chip IMER to a nanovial target plate for MALDI-MS analysis, was reported (Ekström et al., 2000; Bengtsson et al., 2002). The μ-chip IMER was fabricated in silicon and consisted of high-aspect ratio parallel-channel structures with porous surface and immobilized proteolytic enzymes. Enzymatic digestion of proteins was completed in 1–3 min. The microdispenser, with a total internal volume of 1.3 μL, was capable of generating 60 pL–300 nL size droplets with a frequency of 50–100 Hz and deposit the droplets on a high-density nanovial target plate. Alternative approaches for performing on-chip fast proteolytic digestion (3–12 min) involved the fabrication of microreactors with packed immobilized trypsin beads (Wang et al., 2000; Jin et al., 2003), or the integration of poly(vinylidene fluoride) membranes with immobilized trypsin in sandwich-type PDMS devices (Gao et al., 2001).

5. *Microchip Separations Interfaced to MS*

The performance of microfabricated devices with inserted electrospray tips was fully tested for the separation of peptide mixtures and protein digests. Performance similar to that of standard CE (separation efficiency >300,000 plates/m) was demonstrated in 11-cm-long channels fabricated in a microchip interfaced via an integrated liquid junction to a subatmospheric ESI interface-ion trap mass spectrometer, see Figure 10 (Zhang et al., 1999).

Sensitive analysis of tryptic peptides generated either from standard lectins (*Dolichos biflorus* and *Pisum sativum*), or from a band of a 1D-gel separation of membrane proteins from *H. influenzae*, was accomplished using a microchip-CE device interfaced to ESI triple quadrupole or QqTOF systems (Li et al., 1999, 2000b). It was estimated that the analysis was performed from less than 25 ng (1–5 μL sample volumes) of original protein excised from selected gel spots. The starting membrane extract protein mix from *H. influenzae* applied to the 1D-gel was in the

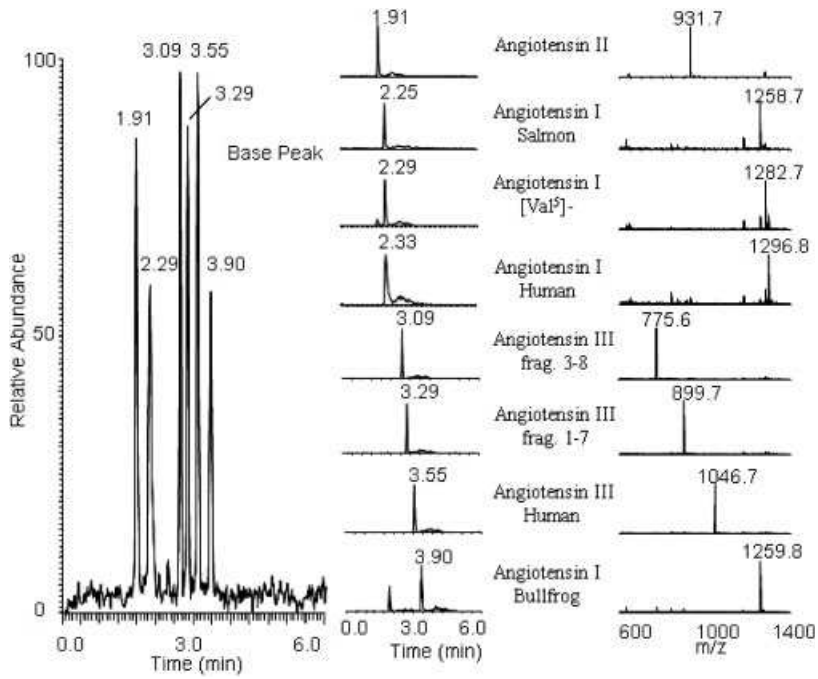

FIGURE 10. Microchip-CE/ESI-MS analysis of a mixture of 10 μg/mL angiotensin peptides using the device shown in Figure 4. Reproduced with permission from Anal. Chem. 2000, 72, 1015–1022. Copyright 2000 American Chemical Society.

1–2 μg range. This low value demonstrates the potential of microchip devices for sample manipulations and analysis at the trace level. Alternatively, on-line protein digestion followed by CE-MS analysis was demonstrated on a microchip platform that integrated an immobilized trypsin bed, a CE separation channel, and an electrospray interface (Wang et al., 2000).

The analysis of small molecules such as drugs and metabolites in human plasma typically requires extensive sample cleanup. Microfluidic systems integrating a CE separation channel with an ESI emitter or a liquid junction microsprayer have the potential to become an alternative to some more established techniques. This potential has already been demonstrated (Kameoka et al., 2001; Deng, Zhang, & Henion, 2001a; Deng et al., 2001b); however, more work will be needed to bring viable products on the market.

Microfluidic CE separations were also interfaced with MALDI-MS detection. The CE separation of oligosaccharides and peptides was performed in open CE channels that contained buffer and MALDI matrix, and after solvent evaporation, the CE channel was scanned with the laser beam (Liu et al., 2001).

Isoelectric focusing of proteins on microchips followed by ESI-MS detection was performed in sandwich-type microdevices prepared from polycarbonate substrates sealed with a polyethylene terephthalate polymer film, that incorporated a laser-ablated separation channel and a pyramid-shaped mechanically machined ESI tip (Wen et al., 2000). Alternatively, CIEF of proteins was performed in poly(methylmethacrylate) chips containing channels that were covered only during the IEF step but not during MALDI, with the matrix solution being added after the focusing step and prior to MS detection (Mok et al., 2004).

A microfabricated CEC device that integrated a double-T injector, a methacrylate-based monolithic separation channel, and an ESI-MS interface, was devised by Lazar et al. (2003) and demonstrated for the analysis of protein digests. Low- or sub-fmol amounts were detected with a TOFMS instrument. The small layout of one CEC processing line enabled the integration of four- to eight-channel multiplexed structures on a single glass chip.

In the recent past, efforts have focused on integrating HPLC systems on the chip, as well. Initially, a silicon substrate was used for preparing a microfluidic channel (20–200 μm in width, 0.5–10 μm in depth, and 15–50 cm in length) that was coated with *n*-octyltriethoxysilane and used for demonstrating the LC analysis of caffeine. This chip was connected to an external pumping/valving system and a UV flow cell for detection; on-chip amperometric detection was provided, as well (McEnery et al., 2000). The integration of bench top LC/MS, fraction collection, and chip-based infusion into one commercial analytical platform was recently accomplished (www.advion.com). This system speeds up the discovery process by allowing extra time for the analysis of complex components, which are difficult to characterize on a chromatographic time-scale. Alternatively, microfluidic LC with mass spectrometric detection was demonstrated from chips fabricated by laminating polyimide films with laser-ablated channels, ports, and frit structures (Fortier et al., 2005; Yin et al., 2005). The enrichment and separation columns on the chip were packed with reversed-phase chromatography particles. These devices were connected to an external rotary valve, a gradient capillary pump, and a microwell plate autosampler for sample loading and mobile phase delivery. This microfluidic arrangement was demonstrated for the analysis of standard protein digests and the identification of plasma proteins. The detection limit of spiked protein digests in rat plasma samples was ~1–5 fmol. This device is commercially available from Agilent Technologies (www.agilent.com).

A fully integrated, stand-alone microfluidic LC device comprised of a multiple open-channel electroosmotic pumping and valving system, a packed LC separation channel, and an ESI interface was fabricated in glass chips (Lazar & Karger, 2002; Lazar et al., 2005). Pressures in excess of 80 psi and flow rates in the 20–400 nL/min range were generated with microchips containing hundreds of ~1.5 μm pumping channels (Fig. 11A). Eluent gradients were created by simultaneously using two pumps. Electrospray was generated from a 20 μm i.d. capillary inserted in the chip, and the system was used for the LC-MS analysis of protein mixture digests and protein extracts from cancer cell lines (Fig. 11B). The system was operated by four power supplies and the miniature format enabled the integration of two complete LC systems on a $1 \times 3''$ chip. Using identical experimental conditions, the performance of the microchip integrated LC system was identical to the one demonstrated from a commercial μ-LC instrument.

6. *Microchip Devices for Multiple Processing*

The small footprint of a microdevice allows facile construction of fully automated analytical systems (Felten et al., 2001; Zhang, Foret, & Karger, 2001). The design shown in Figure 12A consists of three independent modules. The automated sample positioning module (translation stage) is designed to minimize the distance between the sample and analyzer. The analysis module (a microdevice for CE, isoelectric focusing, or chromatography) is interfaced to a mass spectrometer via a subatmospheric electrospray interface (Foret et al., 2000). The microdevice, integrating a sample introduction loop, separation channel, and a liquid junction, is clamped between a manifold of electrode reservoirs, allowing pneumatic sample loading from the microtiter well and flushing with fresh electrolyte after each run (Fig. 12B). The pressure flow control also provides reproducible sample loading without injection bias. An example, shown in Figure 12C, demonstrates MS/MS analysis of selected ion peaks generated during the separation of a BSA tryptic digest. Sample turnaround times of these high-throughput devices were 5–10 sec/sample (Liu et al., 2000; Felten et al., 2001), illustrating the potential of the microfabricated structures for high-speed sample processing.

An alternative microfluidic device comprising a sampling module from an autosampler tray, a sample injector, a CE separation channel, and a nanoelectrospray interface, was utilized for high-throughput sample processing as well (Li et al., 2001, 2002). The device was used for high-throughput on-line sample clean-up/digestion or affinity selection, followed by CE separation and MS detection of submicromolar solutions of protein digests. Sample carryover was estimated to be <3%, concentration detection limits for tryptic digests were ~5 nM (7–25 fmol injections), and the sample processing rate was 12–30 samples/hr. These devices were demonstrated for gel-isolated protein digests from *Neisseria meningitidis* and prostate cancer

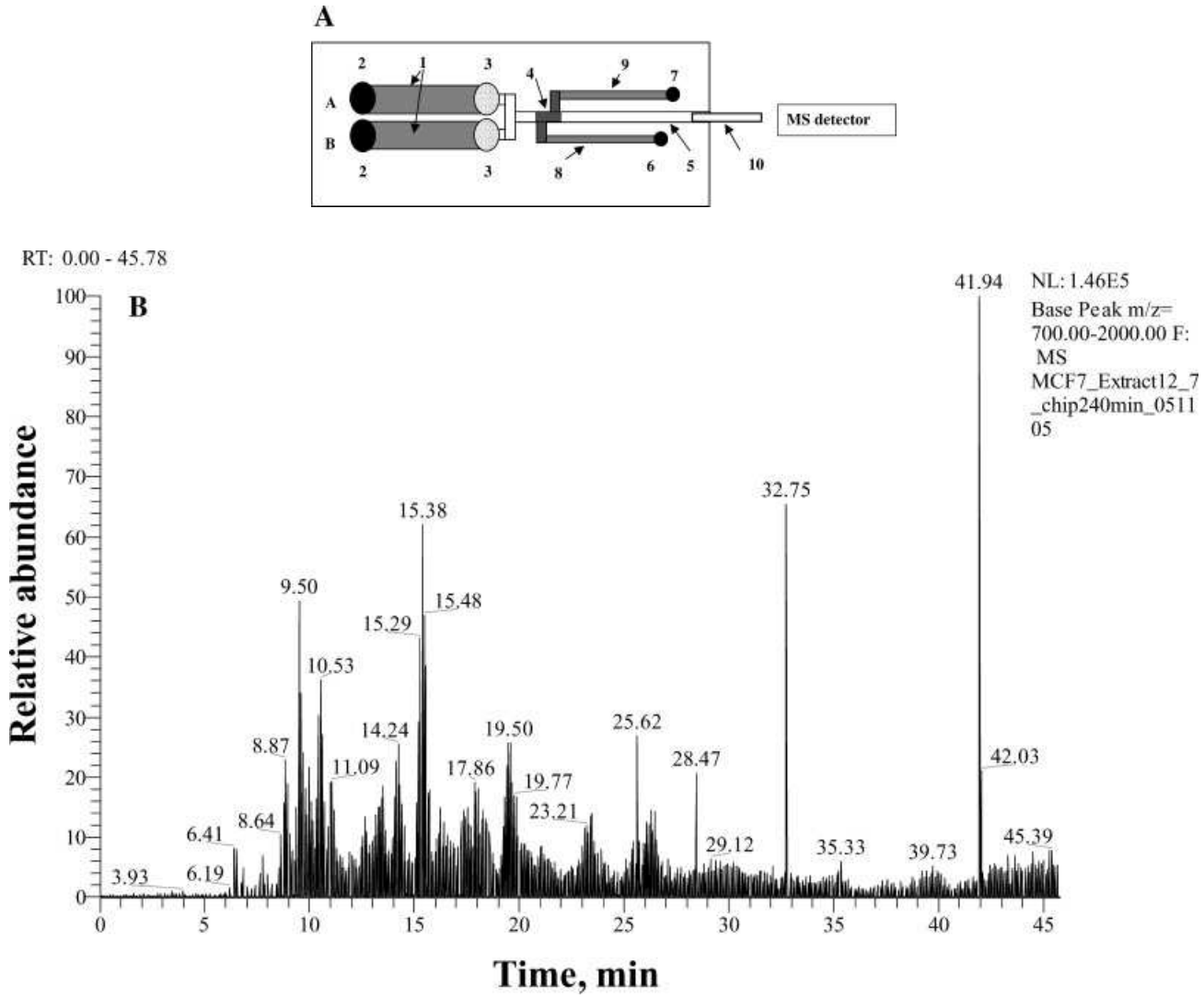

FIGURE 11. Microfluidic LC system. **A**: Schematic diagram of a fully integrated microchip LC device: (1) multichannel electroosmotic pumping system, (2) pump inlet reservoirs, (3) pump outlet reservoirs, (4) sample plug, (5) infusion or LC separation channel, (6) sample inlet reservoir, (7) sample outlet reservoir, (8) sample inlet channels, (9) sample outlet channels, (10) ESI emitter. Reproduced with permission from Anal. Chem. 2002, 74, 6259–6268. Copyright 2002 American Chemical Society. **B**: Base peak chromatogram from a data dependent microchip-LC-MS/MS experiment on a protein digest fraction of the MCF7 cancer cell line.

cell lines, for affinity selected c-*myc* affinity peptides from spiked human plasma, and for IMAC-isolated phosphopeptides.

A microfluidic compact disk integrated with MALDI-MS detection was used for the high-throughput processing of protein digests (Gustafsson et al., 2004). Peptide samples (1 μL) were loaded, concentrated, desalted, and subsequently eluted into MALDI target areas on the CD (200 × 400 μm) with the aid of a solvent containing the MALDI matrix. Fluidic manipulations occurred with the aid of the centrifugal force developed during spinning the CD (∼1,000 rpm). The speed of rotation was adjusted to ensure that the eluate is deposited in the MALDI-target area at a flow rate compatible with the rate of solvent evaporation. The CD was then cut and placed in a commercial MALDI-MS source for detection.

IV. CONCLUSIONS AND FUTURE PERSPECTIVES

The rapid advances of microfluidics are reshaping the bioanalytical world. The newest trends can be now followed in specialized journals such as ''Lab on a chip,'' or dedicated issues of well-established periodicals (Foret, 2004). A more detailed picture of the field can also be obtained from recent review articles on miniaturization (Marko-Varga, Nilsson, & Laurell, 2003; Lion et al., 2004b). This section has shown that much progress has been made in coupling microfabricated devices to ESI and MALDI-MS. The field continues to advance rapidly, and there is good reason to believe that commercialization of new microdevices interfaced to MS will result in the near future. Such devices will offer capabilities for handling very small sample

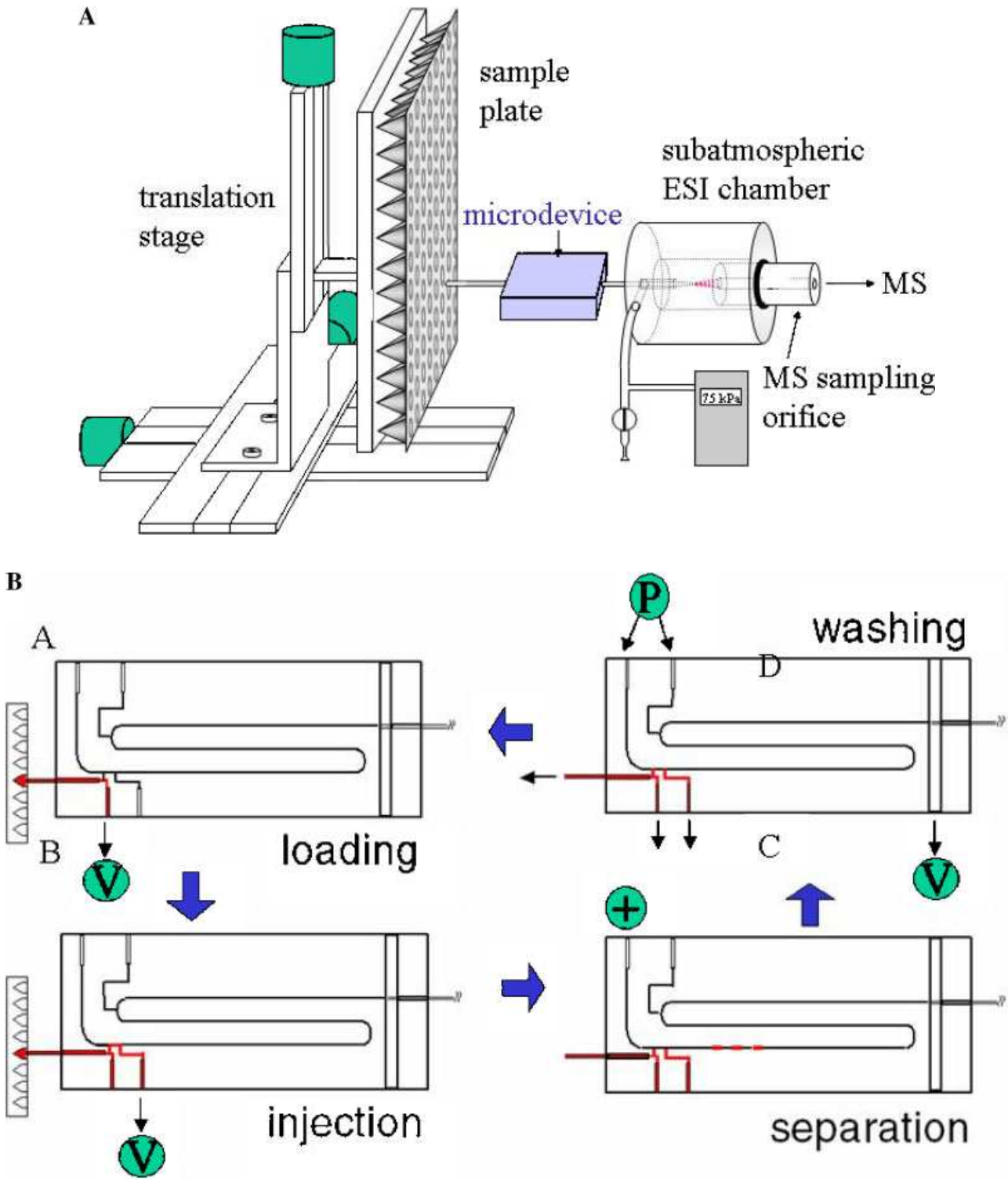

FIGURE 12. An overall system for high throughput separation-MS analysis. **A**: Diagram of the total system. **B**: Schematic representation of all four cycles of analysis (sample loading, injection, separation, and washing) on the microchip. V = vacuum and P = positive pressure. **C**: On-line MS/MS analysis of selected peaks separated on the microdevice shown in Figure 12A. Reproduced with permission from Anal. Chem. 2001, 73, 2675–2681. Copyright 2001 American Chemical Society. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

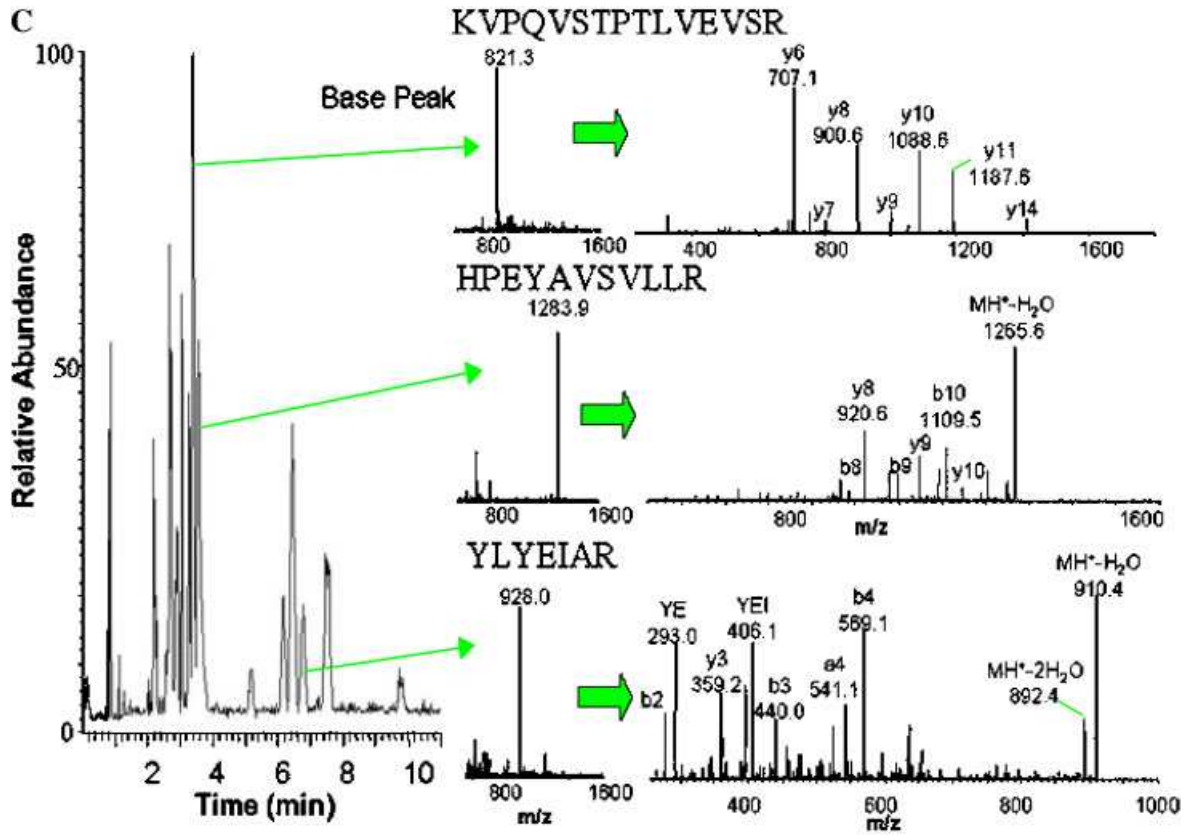

FIGURE 12. (*Continued*)

amounts in relatively short analysis times, and possibly at reduced analysis costs. Microfabrication will inherently enable large-scale integration of entire sample processing lines, multiplexing, and consequently high-throughput analysis. In addition, the miniaturized format will enable the implementation of novel analytical configurations and of physical processes that are not feasible in the macroscale setting. Of course, such devices will be fully automated for successful operation.

The ability to handle limited amounts of cellular protein extracts and to perform detection at the ultratrace level is a challenge in many areas of biological sciences. Microdevices will offer significant advantages, particularly in the context of integrating on-chip sample clean-up, preconcentration and microdigestion prior to separation and mass spectrometric detection. The complexity of some of these samples imposes a need for extensive sample prefractionation for achieving sufficient chromatographic resolution, and ultimately sensitive and efficient MS detection; starting from one protein extract, the protocol may conclude with the analysis of hundreds of sample sub-fractions. Integration and parallelization of analytical processing steps will be essential for promoting high-throughput capabilities, and could potentially result in the fabrication of inexpensive and disposable platforms that prevent sample contamination and carryover, an essential requirement for the reliable analysis of proteomic samples.

Furthermore, the capability to perform high-speed separations with microchips will be fully matched by high-throughput MS instrumentation, especially TOFMS. With respect to separations, CE is now widely utilized on chips and is expected to find extensive applications with MS detection. At the same time, due to robustness, high sample load capacity, and the ability to incorporate hydrophobic separation mechanisms, reversed phase LC will be implemented into microdevices, as well. In the long term, the use of parallel processing for separations and subsequent MS analysis would appear to have significant potential. This seems to be particularly true in the case of multiplexed microfabricated separation devices used in conjunction with MALDI-MS detection. With high-repetition rate lasers, one to two orders of magnitude above those presently used (i.e., kHz), one can envision parallel processing of separations of 10 to more than a 100 channels simultaneously. The development of miniaturized and multiplexed mass spectrometers will further stimulate the need for major advancements in sample processing instrumentation. Such approaches would lead ultimately to ultrahigh-throughput investigations.

A potentially quickly developing area of applications for such microfluidic chips is the fast screening of proteomic samples in clinical settings. High-throughput, contamination-free analysis, and disposability will be essential attributes of the instrumentation that will be utilized for such purposes, and microfluidic chips certainly qualify as successful candidates. A number of companies (Advion, Agilent, Gyros, Predicant, etc.) have already launched efforts to develop novel microfluidic analysis systems with mass spectrometric detection and are

developing bioinformatics technologies for the fast analysis of proteomic and/or clinical samples. Thus, with the forthcoming commercialization of microchip-MS, one can anticipate significant progress in biochemical analysis.

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