A decade of microfluidic analysis coupled with electrospray mass spectrometry: An overview

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This review presents a thorough overview covering the period 1997–2006 of microfluidic chips coupled to mass spectrometry through an electrospray interface. The different types of fabrication processes and materials used to fabricate these chips throughout this period are discussed. Three 'eras' of interfaces are clearly distinguished. The earliest approach involves spraying from the edge of a chip, while later devices either incorporate a standard fused-silica emitter inserted into the device or fully integrated emitters formed during chip fabrication. A summary of microfluidic-electrospray devices for performing separations and sample pretreatment steps before sample introduction into the mass spectrometer is also presented.

Introduction

The way scientists approach chemically related problems has changed significantly since the introduction of lab-on-a-chip or microfluidics technologies at the beginning of the nineties.[1–5] In the early years of microfluidics, most chip-based experiments involved separations, mixing and reactions that were investigated by fluorescence or electrochemical methods.[3–5] However, many of these detection techniques do not scale well with miniaturisation. The sensitivity of a UV absorbance measurement, for example, depends on the length of the detection chamber, a dimension one likes to minimise in microfluidics. Amperometric detection goes with the square of the diameter of a microfluidic channel and becomes therefore also a relatively insensitive detection technique when miniaturised. Mass spectrometry (MS) has become increasingly important as an analysis tool particularly in the proteomics area because of its sensitivity. In addition, of course, MS allows the ionisation of intact molecules for a highly accurate determination of their molecular weight, making identification of molecules easier. A detection limit of 480 molecules has been reported for time-of-flight MS with ions generated on matrix-free porous silicon operated under optimal conditions,[6] whereas this is 18 000 molecules for nanospray Fourier transform (FT) MS.[7]

However, this does not mean that the acquired mass spectrum can be directly translated into quantitative and qualitative information about sample composition, since a large number of chemical and instrumental parameters influence the final appearance of a mass spectrum. From an instrumental point-of-view, parameters such as ion optics and gas pressure in the several stages of the instrument are crucial for the appearance of the mass spectrum (see ref. 8 for a good overview). One critically important process is that of

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ionisation. A critical parameter is the diameter of the electrospray emitter and the flow rate. In the early electrospray ionisation experiments as described by Fenn and others, electrospray emitters with relatively large internal diameters were used (usually on the order of 100 μm) at several μL min^{-1}.[9,10] Droplets that are generated from such emitters are relatively large, requiring a large number of Coulombic explosions before gas-phase ions are formed. Later, 'micro-electrospray' was introduced, operating at sub-μL min^{-1} flow rates with tip diameters around 50 μm.[11] Higher flow rates (mL min^{-1} range) could be sprayed with a coaxially nebulising gas, called 'ionspray' or 'pneumatically assisted electrospray'.[12,13] Mann and coworkers developed a novel way of performing electrospray, called 'nanospray', where small droplets are generated from emitters with an ID of several microns.[14,15] Decreasing the inner diameter (ID) of emitters has several advantages, including lower electrospray voltages that allow the emitter to be positioned closer to the inlet of the MS. The number of ESI-generated ions that are introduced to the MS is increased in this way *i.e.* the yield of analyte ions that enter the MS is higher compared to ESI performed from large-diameter emitters. Nano-ESI consumes less sample, it can be coupled on-line with nano-LC techniques due to the low flow rate, pure water solutions can be sprayed without discharges, and higher ionisation efficiency can be obtained due to the smaller initial radii of droplets generated. Another important reason to use nanospray is that ESI-MS is a concentration-sensitive technique. This implies that the diameter of an emitter can be miniaturised and the flow rate reduced without losing mass spectrometric sensitivity.[16]

An additional advantage of reducing the diameter of the emitter is that ion suppression effects are reduced when going from electrospray to nanospray. The number of analyte molecules per droplet in regular ESI is large and may lead to competition for charges when mixtures of analytes are present. In the case of nanospray, each droplet formed will contain on average a single or a few analyte molecules, eliminating ion suppression effects and making nanospray a very attractive technique. Another reason why nanospray is preferred by many researchers over regular electrospray is that the number of charges available per analyte molecule is much higher in nanospray. This enhances the probability to ionise an analyte.

A drawback of reducing the dimensions of the various components necessary for a successful nano-LC experiment is that the influence of very small dead volumes, normally having a minor impact on micro-LC separations, becomes significant for nano-LC. The quality of the nano connections depends a lot on the experience of the experimenter. Also, variations in tip diameter will have a significant influence on the appearance of the mass spectrum. Microtechnology makes it possible to fabricate chips with sub-micrometer precision and accuracy and with reproducible minimal or zero dead volume connections. It has therefore become an important technology for nano-ESI and nano-LC-ESI experiments. Besides the absence of dead volumes, the main drive for most researchers to work with chips is that multiple functionality such as pumps and valves can be monolithically integrated onto a small device using small sample volumes. Ultimately, chips should be cheap to make and disposable to avoid cross-contamination. Small sample volumes (often sub-μL) are required and as a consequence of the advantageous small dimensions of the channels, analysis can be performed faster, leading to increased sample throughput. Flow rates that are typically obtained in microfluidic channels are on the order of several tens of nL min^{-1}, compatible with nanospray. For these reasons, microfluidics offers researchers a route to new tools to perform experiments that were difficult or not possible to perform previously.

A number of reviews discussing microfluidic analysis coupled with MS have appeared over the years. Early reviews include those by Oleschuk and Harrison,[17] de Mello,[18] Limbach and Meng,[19] and Figeys and Pinto.[20] More recently, reviews focusing on microfluidics-MS application areas such as proteomics,[21,22] carbohydrate analysis[23] and multiplexed analysis[24] have been published, attesting to the rapid growth in this area of microfluidics R & D, Lazar *et al.* published an extensive review on the topic in 2006,[25] while Sung *et al.* chose to focus on microdevices coupled with ESI-MS in a concise review in 2005.[26] A few studies have appeared where microfluidic channels were integrated in MALDI targets,[27–29] and the reviews in refs. 22, 24 and 25 devote some text to this. DeVoe and Lee are probably the first to exclusively review the topic of microfluidics for MALDI-MS.[30] Given this recent review by DeVoe and Lee, applications involving MALDI-MS will not be further discussed in this review. Also worthy of mention but not reviewed here is the transfer of novel ionisation techniques such as atmospheric pressure chemical ionisation (APCI)[31] and atmospheric pressure photo ionisation (APPI)[32,33] to the chip format.

This review presents a thorough overview covering the period 1997–2006 of the state-of-the-art for coupling microfluidic chips to mass spectrometry through electrospray ionisation. Fig. 1 presents the number of publications on this topic as a function of the year of publication. This figure shows that the interest in this subject is still steadily growing. The publications in this figure describe new designs and fabrication processes focusing on the chip and emitter. Commercial products from Advion Biosciences, Agilent and the recent Phoenix S&T have been introduced on the market and have proven to be very successful for a wide range of sample types.

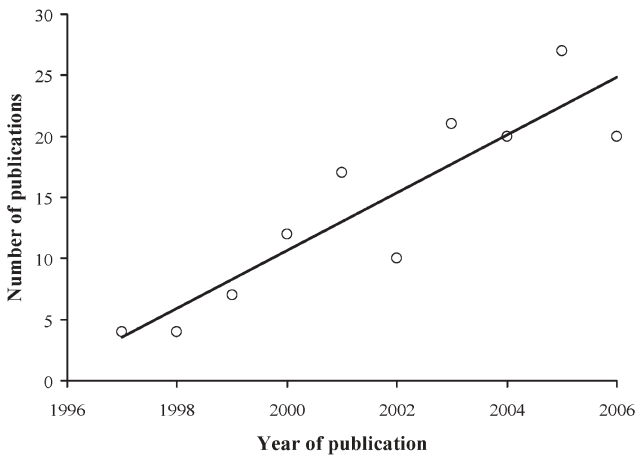

Fig. 1 Number of publications dealing with the coupling of microfluidic chips to mass spectrometry by ESI as a function of the year of publication.

Publications making use of especially Advion Biosciences chips are numerous, focusing more on application than on modifications of the chip itself. Only the first publications on this chip are therefore included in the figure. An overview of publications making use of the Advion Biosciences chip can be found elsewhere.[34] Had these publications also been included, an exponential curve would have been observed in Fig. 1.

The first part of this review briefly describes the currently most commonly used fabrication processes and materials to make chips. The second part describes the first approaches to couple chips to mass spectrometry where spraying was performed from the edge of the chip until the latest developments where electrospray emitters are fully integrated in the fabrication process. In the third part, the potential of multiple emitters integrated on a single chip is explored and discussed. The last part of this review is an overview of different types of separation, sample preconcentration and sample clean-up experiments that have been performed on an ESI chip.

Materials used for chips and chip fabrication processes

The microfluidics community has adopted a number of cleanroom microfabrication processes used in the semiconductor industry since the early 1960s to micromachine several classes of materials to fabricate chips. These processes were originally optimized for silicon, the material of choice for microelectronics components such as transistors, and so many of the early microfluidic devices consisted of microchannels made in silicon. However, the same basic photolithographic patterning techniques have also been successfully applied to microstructuring planar glass and quartz surfaces, as well as the formation of microchannels directly in photoresist. During the first decade of lab-on-a-chip research (1990–2000), the interest in glass as a substrate material for fluidic applications grew rapidly. This is because it is chemically inert, has well-defined surface characteristics, and can be used to generate electro-osmotic flow (EOF). The popularity of silicon declined during this period, in large part because it is not compatible with the use of high electric fields for generation of EOF. However, silicon continues to be used for applications employing pressure-driven flow and/or complex microstructures for which processing techniques in alternative materials do not yet exist. Advion Biosciences for example, has an ESI chip on the market made of silicon, based on an array of micronozzles.[35] Plastic substrates have recently started to increase in popularity. Agilent has introduced an ESI chip made of polyimide by laser ablation.[36] Phoenix S&T has an ESI chip on the market made of polypropylene by injection moulding.[37] Tables 1 and 2 present an overview of all materials that have been used to make electrospray chips and emitters and the technologies used for micromachining, respectively. Table 2 makes a distinction in technologies between cleanroom-intensive and non-cleanroom approaches. Cleanroom-intensive technologies, such as silicon micromachining, require dust sensitive processes. Techniques such as hot-embossing do not necessarily require a cleanroom, although the master may need to be fabricated in a dust-free environment.

Table 1 Materials that have been used to fabricate ESI-MS devices

Chip material	References
Glass/Pyrex	25,31,32,38–43,45–74,83,153,156
Quartz	44,157,158
PDMS	103–115,125,126,159–162
PMMA	95,102,117,125,151,154,163–165
Polycarbonate	102,121,127–130,132,149,155,166
Polyimide	131,135,136,142–148,167
Polyester	126,130,133,134,137–148,150,152,168
Zeonor	116,118–120,122–125
SU-8	85–94
Silicon	31,32,72–84,96,100
Polysilicon	97,98
Glassy carbon	101
Resin	169
Parylene	96,99
Silicon dioxide	78,81

Table 2 Materials and technologies used for ESI-MS devices. The processes mentioned in the table as such do not require a cleanroom although areas with a lot of dust may cause problems especially during bonding of two plates together. For example, the fabrication of the master for PDMS replica moulding requires cleanroom processes. The replica moulding process itself can be performed outside the cleanroom

Fabrication technology	References
Cleanroom intensive	
HF etching	25,31,32,38–74
DRIE	74–84
UV exposure/SU-8	85–88,89,90–94
X-ray photolithography	95
Other MEMS processes	96–100
Electrochemical anodization	101
Anisotropic etching	31,32,96,102
Non-cleanroom approaches	
Replica moulding PDMS with SU-8[a]	103–109
Replica moulding PDMS with capillaries, wires or other surfaces	110–115
Hot embossing[a]	116–125
Embossing[a]	126,127
Laser ablation	128–141
Plasma etching[a]	142–146,147,148
Injection moulding	102,149
Laser printer	150
Micro milling	151,152
Other mechanical techniques	153–155

[a] May require cleanroom processes.

Chip based electrospray ionisation sources

Several methods have appeared in the literature for coupling a microfluidic chip to a mass spectrometer, using electrospray as ionisation technique. These can be roughly divided into three general approaches. The first approach, reported in the late nineties, was monolithic, with electrospray being performed directly from microfluidic channels opened at the edge of a chip. Though this approach is simple, the position of the electrospray along the chip edge is often difficult to control. To overcome this problem, various groups devised a second approach, which involved inserting fused-silica capillaries directly at the ends of channels in a chip, to serve as electrospray tips. This approach is technically difficult to realize, however, inspiring researchers to try yet another approach, which was to monolithically integrate emitters in the

fabrication process. These three generations of chips are described in this section together with some illustrative examples. The advantages and disadvantages of each approach will be discussed.

Spraying from the edge of a chip

The first groups to explore the possibility of coupling on-line analysis in microfluidic chips with mass spectrometry were those of Karger *et al.*[41] and Ramsey and Ramsey.[45] Both reported, at about the same time, microfluidic chips that allowed electrospray ionisation to be performed from the edge of the chip.

Karger *et al.* were far ahead of most groups because in addition to being the first to couple chips to ESI-MS, they were the first to fabricate chips with multiple channels from which different samples could be analysed. They reported a glass chip with nine microfluidic channels for direct infusion experiments.[41] This device is shown in Fig. 2 and has 60 μm-wide and 25 μm-deep channels. The spray was formed directly from the edge of the chip using an external syringe pump to generate a pressure-driven flow of 100–200 nL min^{-1}. One of the main issues with this device, however, was that the liquid leaving the microfluidic channel tended to spread along the edge of the chip. Hydrophilic liquids have a low contact angle with glass and will tend to easily wet glass surfaces. To prevent this from happening, the surface was coated with a hydrophobic reagent, n-octyltriacetoxysilane, also known as imunopen. A detection limit of 60 nM for myoglobin was observed. Later, the same authors demonstrated the mass spectrometric analysis of a tryptic digest of melittin performed in the reservoirs on the chip.[42] Their chip did not permit an electro-osmotically driven flow (EOF) in the set-up reported, because the current flowing through the microfluidic channels was limited by the ESI process to around 100 nA (see Fig. 3a). This is at least one order of magnitude too low to obtain sufficient EOF in typically used buffer systems.

Ramsey and Ramsey used a chip with 10 μm-deep and 60 μm-wide channels in which pumping was performed electro-osmotically.[45] The chip contains a main channel with an exit in the edge of the chip where electrospray is generated, see Fig. 2b. A side channel or arm is connected to the main channel near the exit. An ionic current high enough to induce an electro-osmotic flow in the main channel is generated through the channels by applying a 4 kV potential difference between the buffer inlet reservoir and side-arm reservoir. EOF in the side channel is suppressed by a polyacrylamide coating that increases the surface viscosity and reduces the zeta potential of the glass walls. The coated side-arm therefore cannot accommodate all the EOF generated in the uncoated main channel, resulting in an increase in pressure at the intersection of the main channel and the side-arm. The side-arm channel is longer than the remainder of the main channel to the channel opening, and thus has a higher resistance to flow. This, combined with the difference in EOF capacity of the two channels, results in an indirect pressure-driven flow towards the channel opening in the edge of the chip. This flow is high enough to obtain a constant electrospray current. A schematic overview of the electrical configuration of the chip is shown in Fig. 3b. Another electrical configuration that has only been used once with edge emitters is presented in ref. 69. A thin glass membrane above the channel was integrated on the chip to apply the potential. This electrical configuration is not included in Fig. 3 but shows similarities with Fig. 3f, which is discussed in more detail in the next section, "Spraying with inserted capillaries".

Although the fabrication of these chips with spray orifices directly on the edge is relatively simple and truly monolithic in nature, several disadvantages with this approach make it unsuitable for a number of applications. First of all,

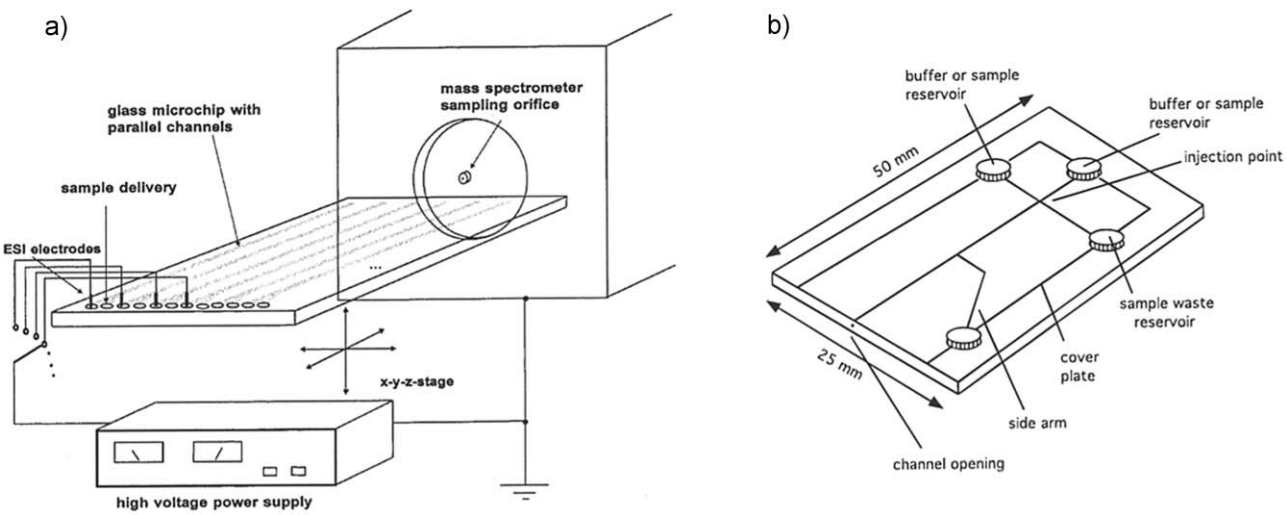

Fig. 2 (a) Schematic diagram of the micro-chip ESI-MS interface of Karger *et al.*[41] Each of the nine microfluidic channels has two inlet wells. One well is for the sample and can be connected to a syringe pump. The second well contains buffer that can be connected to a high-voltage power supply. The exit ports of the microchip were aligned with the orifice of the mass spectrometer using a 3D translational stage. (b) Diagram of the channel layout for indirect pumping induced by electro-osmotic flow. The sample and side-arm reservoirs were set at 6 and 2 kV, respectively. The channel opening where electrospray is performed was held at a potential of 4.8 kV. The side-arm channel was coated with polyacrylamide, to suppress EOF.[45] [(a) Reprinted with permission from ref. 41. Copyright 1997 American Chemical Society. (b) Reprinted with permission from ref. 45. Copyright 1997 American Chemical Society.]

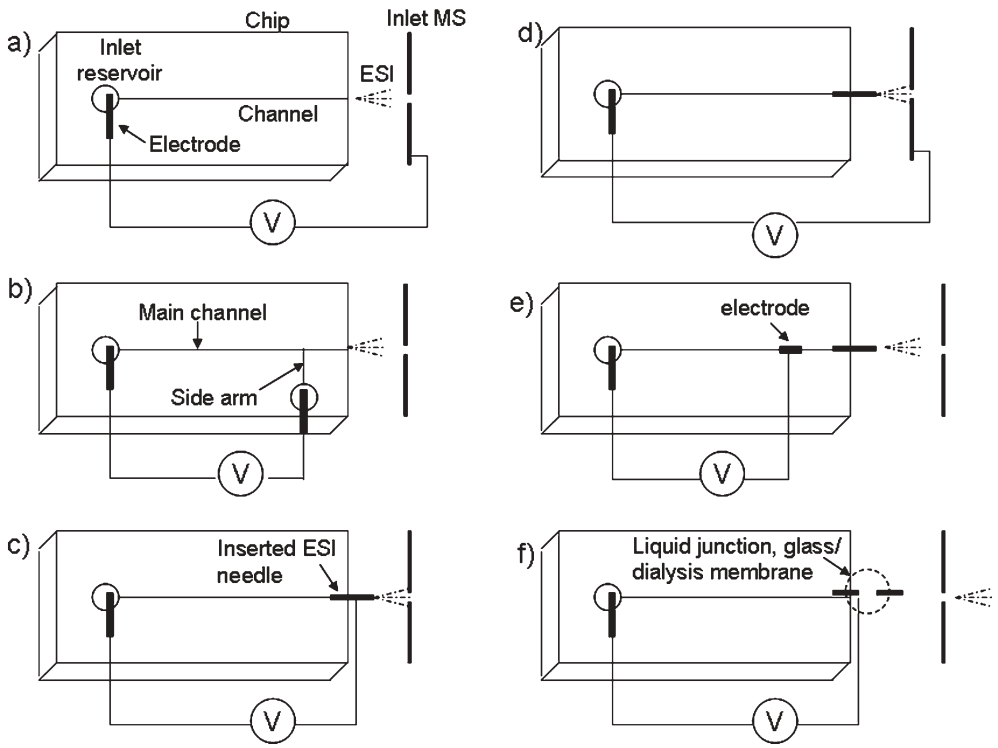

Fig. 3 Different electrical configurations of ESI sources integrated on a chip: (a) Spray directly from the edge of a chip, with the spray potential applied between a reservoir and the inlet of the mass spectrometer *e.g.*, ref. 41; (b) spray from the edge of a chip, with potential applied between inlet reservoir and coated side-arm channel at the end of the main channel which can serve as a separation channel *e.g.*, ref. 45; (c) spray from an inserted fused-silica (FS) needle, with the potential applied between the reservoir and the FS needle *e.g.*, ref. 47 and 48; (d) as (c) but with the potential applied between the reservoir and the inlet of the MS *e.g.*, ref. 54; (e) electrode integrated in microfluidic channel for applying the electrical field for ESI[144–146,148]; (f) spraying with an integrated liquid junction or dialysis/porous membrane.[53,55,112,126,130]

hydrophilic liquids have a low contact angle with the hydrophilic glass, and therefore tend to spread along the edge of glass devices. The same is true for hydrophobic liquids, which wet hydrophobic chips made of plastics. This makes it difficult to predict the exact position from where the spray is generated. Small sharp objects, such as small splinters of substrate material left on the side of the chip after fabrication, experience a high local electrical field strength, as reported by Rohner *et al.* for PET/PE chips.[134] Moreover, the total volume of liquid wetting the edge of the chip is relatively large and may act as a dead volume to negatively influence the performance of separation chips.[134] To avoid spreading of hydrophilic liquids, hydrophobic coatings have successfully been tested[41,42] but were found not to be stable over time. Coatings are also not a solution for the wetting problem for on-chip separations performed in gradients of solvents with different polarities. A possible approach to overcome some of the problems described here is by closing the exit of the microfluidic channel with a porous PTFE membrane from which small Taylor cones are generated.[121] Similarly, the formation of a porous monolithic phase in the emitter can lead to stable electrospray from the end of the emitter.[67,170]

Spraying with inserted capillaries

To overcome the problems that arise when spraying from the edge of a chip, several researchers have described connecting standard fused-silica emitters to microfluidic channels.

Harrison and coworkers were among the first to describe a novel approach to integrate a fused-silica electrospray emitter with their chip.[63] Glass devices for CE separations were made and 200 μm-holes were drilled in the edge of the chip using a tungsten carbide drill, see Fig. 4a and b. To avoid clogging the channels with glass particles, they were first filled with a glue that could be removed after the drilling process by heating to its melting point. The glue was also used to seal the connection between chip and needle.

A capillary with an outer diameter (od) of 185 μm and inner diameter of 50 μm was then inserted into the hole, connecting with the 13 μm-deep and 40 μm-wide microfluidic channel. The influence of the connection on the CE performance, *i.e.* the plate number, was studied by separating a mixture of fluorescently labelled amino acids using laser-induced fluorescence as detection technique.

Two chips were compared, each with a different dead volume at the channel–capillary junction due to differences in the drill bit geometries used. One of the drills was pointed, shown in Fig. 4b, and the other, flat-tipped. When the hole was drilled using a pointed drill, a dead volume of 0.7 nL resulted, which in turn meant broader peaks (see Fig. 4c for a picture of the connection between chip and fused-silica emitter). Plate numbers for the separation of fluorescently labelled amino acids were measured to be 40 000 on the chip at the end of the microfluidic channel, *i.e.* the spray capillary not taken into account. When the same compounds were separated on the

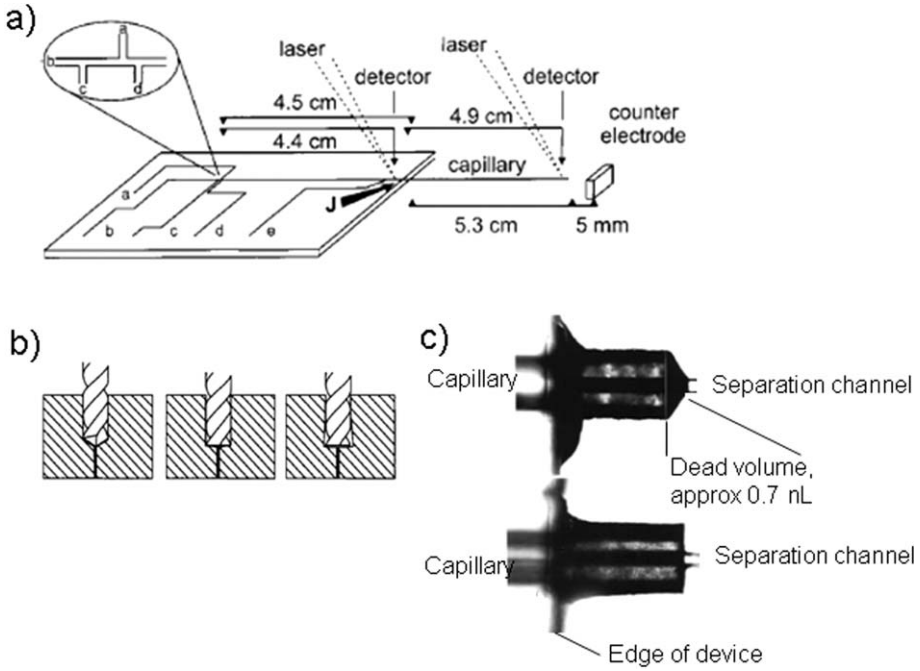

Fig. 4 (a) Glass chip with capillary inserted into a hole drilled in the side of the chip. (b) Procedure to drill tapered and flat-bottomed holes into glass. The geometry of the hole is dependent on the shape of the drill bit. (c) Photos of fused-silica capillaries inserted into holes drilled into the edge of the device. Both pictures were taken looking down onto the upper glass surface. In the upper photo, the hole was drilled with a pointed drill; the resulting dead volume is about 0.7 nL. The lower photo shows a capillary butt-connected directly to the end of the microchannel in a hole drilled with a flattened drill. The intermediate dead volume between emitter and channel is negligible.[63] [Reprinted with permission from ref. 63. Copyright 1999 American Chemical Society.]

device and detected at the end of the inserted electrospray needle, plate numbers around 15 500 were obtained, even though the separation length was almost doubled. Better results were obtained with the flat-tipped drill where the inner capillary butted right up against the microfluidic channel, leading to a negligible dead volume. In this case, the plate numbers for the separation of the same compounds were 117 000 at the end of the microfluidic channel and 71 000 at the end of the inserted spray needle. This study demonstrated very clearly that integrating electrospray emitters in chips requires special attention. When a similar chip was coupled to a mass spectrometer by this group for the analysis of low nanomolar peptide mixtures,[47,48] a configuration as shown in Fig. 3c was used.

Furthermore, the system was used for the identification of unknown proteins isolated from the gel electrophoresis of extracts from *H.influenzae*. The surface of the channels were coated with [(acryloylamino)propyl]trimethylammonium chloride to prevent adsorption of analyte onto channel walls.

A similar approach to connect fused-silica capillaries with glass chips was followed by Zhang *et al.*[40] who inserted the capillary in a 400 μm-diameter HF-etched cylindrical cavity connected to the 75 μm-deep and wide separation channel. The separation channel and cavity were etched in two glass structures which were fusion-bonded together to generate circular channels. A fused-silica capillary having a 380 μm od was inserted in the cavity and epoxy glued to avoid leakage.

The above method to couple emitters with microfluidic devices relies on applying the high voltage to a metallic coating on the emitter, a configuration which is schematically shown in Fig. 3c.[171] In another approach, the electrical field is applied between one of the inlet reservoirs on the chip and the inlet of the mass spectrometer, as shown in Fig. 3d.[54] Note that this works well for applying the electrospray potential, however, when separations based on CE need to be performed, this approach will not work due to the electrical current limitation imposed by the ESI process, as explained in the previous section. An exception to this has been demonstrated by van der Greef and coworkers, who obtained a stable spray and good separation of β-agonists by applying the potential at one of the inlet reservoirs using the inlet of the mass spectrometer as counter electrode.[127] They made use of a low-conductivity buffer that exhibits a large potential drop over the microfluidic separation channel, enabling separation by CE. However, it is frequently not desired to use a low-conductivity buffer when performing separations. This is because a large potential difference between the end of the microfluidic channel and one of the inlets of the microfluidic device would be needed to perform a separation, resulting in the generation of heat. In other approaches, the electrical field is applied to a stainless-steel tube connected to the exit of a chip through which the eluent is guided[59,60,165] or to electrodes integrated in the microfluidic channel, which may be made of gold,[144–146,148] conductive carbon[133,134] or conductive epoxy,[169] see Fig. 3e. All these approaches have the advantage that compounds eluting from the column are directly electrosprayed without dilution.

A different configuration consists of a liquid junction frequently making use of a sheath flow of make-up buffer.

This approach has the advantage that the composition of the separation buffer can be modified to make it suitable for ESI. Also, when the flow rate from an external LC column is incompatible with the ESI source used, a make-up solvent can be added,[172] though dilution of the analyte will take place. Most liquid junctions are made by inserting a spray capillary into the device and coupling it to an existing ion source.[38,39,44,52,61,62,107,118,157,173] Couplings where the potential is applied to a side channel in contact with the separation channel through a porous (glass) membrane,[53,55,69,130] or a dialysis membrane made of polysulfone[112,126] (see Fig. 3f) have also been reported.

The coupling of (nano)-electrospray emitters with microfluidic channels improve the ESI-MS performance significantly compared to spraying from the edge of a chip. This approach has also allowed microfluidic devices to be coupled to commercial electrospray ion sources. However, several difficulties using this approach should be mentioned. Dead volumes, having a negative effect on the separation performance, are easily introduced upon coupling, though they can be avoided using complex drilling approaches involving gluing.[63] These drilling procedures may not be the best choice if a microfluidic device needs to be disposable and therefore cheap to fabricate. Moreover, these approaches tend to be subject to failure. Liu and coworkers, for example, demonstrated a chip consisting of a 96-well plate where each well was connected to an individual microfluidic channel and a manually inserted fused-silica spray needle.[169] Although their work was a nice example of high-throughput analysis, 7 out of the 96 sprayers failed to spray. This was probably due to an error during the gluing process required to make the well plate/fused-silica capillary connection fluid-tight. Manually inserting spray needles in chips is, from a labour-intensity and cost point-of-view, not the cheapest way to make devices. Additionally, though not reported, glue may release compounds that interfere in the mass spectrum under certain buffer/organic solvent conditions. The next section will describe a third approach to make chips where the electrospray emitter is made during one of the chip-fabrication process steps.

Integrated emitters

As discussed in the previous section, one of the main problems arising with manual insertion of spray needles into microfluidic channels is that the approach does not lend itself to mass fabrication.[169] Two approaches have demonstrated that chips can be made in a highly integrated fashion where both microfluidic channel and emitter can be formed simultaneously during the same fabrication process, eliminating dead volumes. The first approach makes use of technologies that allow fabrication of the microfluidic channel and emitter in a single machining step, one chip at a time *i.e.* serially. Examples are based on laser machining and milling. The second approach makes use of photolithographic processes that allow large numbers of structures to be made in a single step and can therefore be used to make many chips simultaneously. This approach is more parallel in nature. Examples of chips made using these approaches are discussed below.

Serial fabrication of chips with integrated emitters

Rohner and coworkers scanned an excimer laser over the surface of a poly(ethylene terephthalate) film to make microfluidic channels. The chip was cut out from the sheet using the same laser. During this cutting process, the shape of the emitter was defined.[134] A carbon-ink electrode was integrated onto the device to apply the high voltage for ESI. In a later publication, they report a similar device where a carbon-ink electrode was used to electrochemically tag free cysteine residues in proteins. Electrochemical oxidation of *p*-hydroquinone was performed at the electrode to generate *p*-benzoquinone. This product reacts with the free cysteine residues of proteins in the microfluidic channel, and was measured successfully with ESI.[133] The approach allows one to identify the number and position of the cysteine residues in proteins. Tang and coworkers used an excimer laser to drill 30 μm holes through a 1 mm-thick polycarbonate sheet.[132] An array of nine sharp electrospray emitters in the form of nozzles is formed on one side of the sheet, similar to the nozzle arrays presented by Schultz and coworkers in silicon using deep reactive ion etching (see next section).[79] Liquid is supplied from the other side of the sheet to generate nine Taylor cones that are directed to the MS simultaneously. An increase in mass spectrometric sensitivity by a factor of 2–3 was observed compared to standard pulled capillaries.[132]

Van de Goor and coworkers from Agilent presented a chip that exhibits several novel features.[131] The microfluidic channels and spray tip of their multi-layer polyimide chip are made using laser ablation. The chip is sandwiched between the stator and rotor of a 2-position rotary switching valve normally used for HPLC. This enables a robust and reliable coupling of the macro world, using a nano HPLC pump, to the micro world. The chip contains two columns, of which a trap column is located in the rotating part of the valve, as shown in Fig. 5. In an initial study, sample was introduced at 4 μL min^{-1} with a standard LC pump. A 1 μL sample of peptides from a 20 fmol μL^{-1} tryptic digest of BSA was trapped on the enrichment bed between the stator and rotor containing 5 μm

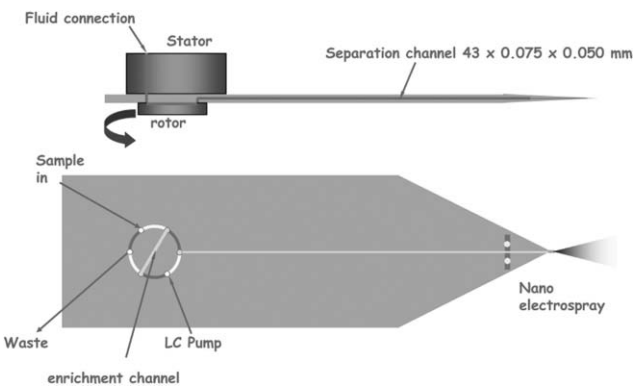

Fig. 5 Polyimide chip sandwiched in between the stator and rotor of a two-position HPLC rotary switching valve. The enrichment column is centred in the rotary switching valve. A sample is loaded onto the enrichment column to trap the analytes of interest. Injection on the LC column is performed by switching the rotary valve and eluting the compounds in a LC gradient.[167,174] [Reprinted with permission from ref. 174. Copyright 2006 John Wiley & Sons Limited.]

C18 particles. By switching the valve and eluting the trapped peptides from the enrichment column, the sample could be injected onto the analytical column containing 3.5 μm C18 particles and separated by applying a reversed-phase gradient at 100–300 nL min^{-1}. Results comparable to standard state-of-the-art nano-LC-MS were obtained.[131,167] Later, a 2-dimensional LC separation was performed with the same chip by another group. An off-chip but on-line SCX column was used to trap the peptides from tryptic digests and plasma. Peptides were sequentially eluted onto the C18 precolumn with different concentrations of ammonium acetate at pH 3.5. The trapped peptides were eluted and separated in reversed-phase mode using a gradient of H_2O/ACN/FA.[135]

Parallel fabrication of chips with integrated emitters

Kim and coworkers made a 16-channel, PDMS microfluidic chip with integrated emitters using a template made of SU-8.[103] A single photolithographic exposure of the photoresist was required to structure the master for replication of the 16 channels. The observed sensitivity of the PDMS emitters is lower than the sensitivity of pulled nanospray needles from fused silica, but a stable spray could be maintained for over 30 h.[103–105] Care should be taken when using PDMS, however. When exposed to organic solvents, PDMS may release small oligomers and additives that interfere in the mass spectrum. This was not observed by Chan and coworkers,[107] who used a 10% methanol spray solution. In contrast, Huikko and coworkers report significant chemical background when using a 50% methanol spray solution.[106] The chemical background could be significantly reduced by increasing the curing time of the PDMS from 6 h at 70 °C to 48 h at 70 °C.

Other researchers have realized emitters in the SU-8 photoresist itself, with geometries based on an open-structured needle shape resembling a fountain pen. SU-8 lends itself perfectly for mass fabrication because it can be patterned by photolithography. The sample to be analysed is pipetted into a reservoir integrated with the SU-8 structure and flows through a connecting open channel to the tip of the SU-8 structure (8 and 16 μm wide). Mass spectra were recorded for peptide concentrations down to 1 μM. The 8 μm tip showed a better performance than the 16 μm tip.[85–88] More recently, this same group presented an SU-8 chip into which a monolithic phase was integrated for the trapping and purification of peptide samples.[175,176] They have also demonstrated SU-8 tips coated with nickel, to make them conductive, and SiO_2 to improve the hydrophilic character of the tip.[89] Similar but smaller tips, with 1.8 × 2 μm and 2.5 × 5 μm tip dimensions, have also been made from polysilicon.[97,98] Others have presented fully enclosed SU-8 tips.[92]

Kameoka and coworkers have reported a chip containing four microfluidic channels made by hot embossing in Zeonor polymer, a thermoplastic polyolefin resin which is becoming increasingly popular with microfluidics researchers.[119] The spray tips are made of 5 μm-thick parylene film and have a triangular structure. In contrast to other studies, the parylene tip does not contain a channel but rather a sharp triangular point where the Taylor cone is formed. This parylene triangle is sandwiched between the plate containing the microfluidic channel and a flat piece of Zeonor. It serves to guide the liquid to the tip of the triangle for ESI. The high voltage necessary to maintain a steady electrospray was applied to the reservoir connected to the microfluidic channel. Stable Taylor cones and ESI-generated currents were observed without cross contamination. More recently, the same group demonstrated the integration of Au electrodes on the Zeonor polymer to supply the high voltage required in the ESI process. Stable Taylor cones were observed and the precision (3.2% at 200 ng mL^{-1}) and accuracy (101.2% at 200 ng mL^{-1}) of direct infusion experiments performed with the biologically relevant molecule methylphenidate extracted from urine were good. This chip has been used for quantitative analysis of methylphenidate by making use of an internal standard.[122]

Simultaneous fabrication of multiple chips incorporating both channels and emitters is also possible when using silicon and glass. Kostiainen and coworkers presented a glass/silicon chip with a single microchannel and integrated emitter for both atmospheric pressure chemical ionisation (APCI)[31] and atmospheric pressure photo ionisation (APPI)[32,33] experiments. The chip consists of a glass/silicon stack with an aluminium heater deposited on the glass surface for APCI measurements and channels etched into the silicon wafer. A significant enhancement of the ionisation efficiency was observed by heating the microfluidic channel from room temperature to temperatures between 60 and 80 °C for molecules with a fairly high boiling point. Decomposition at higher temperatures was observed for specific compounds. The chip could be operated at a wide range of flow rates (50 and 5000 nL min^{-1}) compatible with both nano- and micro-electrospray. The detection limit under APCI conditions is comparable to commercial instruments. An additional repeller close to the chip was needed for the APPI experiments to transfer the ions made by a 10 eV krypton discharge lamp to the mass spectrometer in a suitable electrical field. The mass spectra obtained for small standard compounds were similar to spectra obtained with commercial instruments, and devices could be operated for weeks.

Several authors have demonstrated the micromachining of polyimide using laser ablation. Rossier *et al.* have, however, patterned the material in a plasma. This has the advantage that a large number of features are made simultaneously, in contrast to laser ablation.[142–146] Arrays of hundreds of emitters have been fabricated by Zhang and coworkers by etching through silicon wafers with deep reactive-ion etching (DRIE).[79] The nozzles had a 20 μm od and a 10 μm id, which yielded a corresponding 25 pL volume. A large area is etched away at the back of the wafer to allow a capillary to be connected to a nozzle for sample introduction. The performance of the nozzle arrays is very good, with a S/N of 450 for a 10 nM cytochrome C solution in 100% water at 100 nL min^{-1}. Sensitivity was 1.5–3 times higher compared to pulled-capillary emitters. A much more stable ion current was also possible (~2% RSD compared to ~10% RSD for standard pulled nanospray tips). The lifetime of the emitters was about 8 h for continuous operation.

A chip with an array of 10 × 10 of these nozzles was used in conjunction with a 96-well plate. Each sample could be introduced to an individual ESI needle[75,76] from the backside of the chip using disposable conductive pipette tips. After

positioning the pipette tip at the backside of the ESI chip, nanospray was induced by applying a small pressure together with the spraying potential. The influence of salts on peak intensities in the observed mass spectra was studied, and found to be small for peptides from tryptic-digested proteins dissolved in 100 mM ammonium acetate and 0.0125% SDS. Analysis of proteins measured in pure methanol with 0.1% acetic acid, in 100% water, and in several water–methanol ratios was performed. Although protein unfolding was observed by a shift in the protein-charge-state distribution, successful mass spectra were recorded for all solvent compositions. A detailed characterisation of the accuracy and precision of the chip was performed in a later study.[80] The inter-assay precision was <5%, intra-assay precision <16% and overall accuracy 9%, as determined with the pharmaceutical compound, midazolam. Analyte carry-over was not observed. Other studies with the same chip have been performed for applications as varied as proteomics, glycomics and phosphorylation,[177] pharmaceutical analysis,[77,82] noncovalent interactions,[178] lipid analysis,[179] metabolite identification,[180] biomarker discovery[181] and small molecule quantification.[82] These nozzles, first reported by Schultz *et al.*,[79] have gone on to form the basis for a commercially available chip-based ESI-MS interface.[35]

Fig. 6 shows a few examples of integrated emitters made using several types of cleanroom processes. See figure caption for details.

High-throughput analysis

Parallel analysis for high throughput is of interest to the analytical chemist for economical reasons. However, a mass spectrometer can in principle handle only one analysis at a time. A way to work around this has been presented by de Biasi and coworkers, who developed a four-channel electrospray interface for the analysis of the compounds eluting from four LC columns.[182] Four separations are performed simultaneously, but the eluates are analysed in a multiplexed manner. This can of course have the disadvantage that compounds eluting from one column may not be registered while mass spectra are being acquired from another column. Similar chip-based experiments in which multiple channels are monitored simultaneously using an MS have not been performed. However, several studies do describe the use of multiple channels integrated on a chip, each connected to an individual nozzle, for high-throughput analysis in a serial fashion (one channel at a time). These devices have the particular advantage that cross-contamination is avoided, since each channel/nozzle is used for only one sample. The first chip with multiple

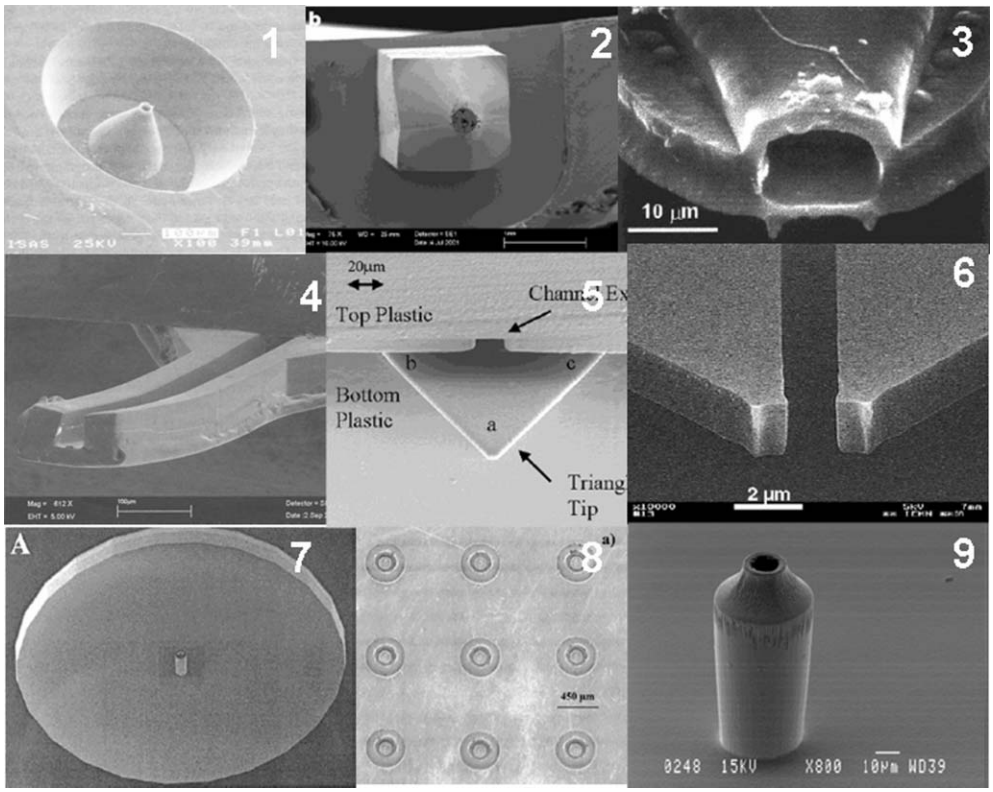

Fig. 6 A selection of integrated emitters. Emitter 1 is made in polymethyl methacrylate by micromilling,[151] 2 is made in polycarbonate/polymethyl methacrylate by injection moulding,[102] 3 is a parylene emitter,[96] 4 is a PDMS emitter made by replica moulding,[113] 5 is an emitter formed from a triangular piece of parylene sandwiched between two Zeonor substrates structured by hot embossing,[119] 6 is an emitter made in SU-8 using standard cleanroom processes,[97] 7 is an emitter made in silicon using deep reactive-ion etching,[79] 8 are nozzles made in polycarbonate using laser ablation,[132] 9 is made in silicon dioxide using deep reactive-ion etching.[78] [(1), (4) Reprinted with permission from ref. 151 and 113. Copyright 2004 Royal Society of Chemistry. (2), (3), (5), (7), (8) Reprinted with permission from ref. 102, 96, 119, 79, and 132. Copyright 2003, 2000, 2002, 2000, 2001 American Chemical Society. (6) Reprinted with permission from ref. 97. Copyright 2005 Elsevier. (9) Reprinted with permission from ref. 78. Copyright 2003 John Wiley & Sons Limited.]

channels/emitters, presented by Karger *et al.*,[41,42] contained nine parallel channels, each spraying from an individual orifice at the edge of the chip, see Fig. 2.

Figeys presented a chip containing nine parallel microfluidic channels, of which the outlets were all connected to one off-chip micro-ESI ion source. One of the reservoirs contained a buffer that was used to rinse the off-chip micro-ESI source after each MS analysis. Cross-contamination of the tryptic-digested proteins could be minimised in this manner. The samples were pumped through the channels by electro-osmosis, with potential application being controlled by a set of high-voltage relays.[59]

Liu and coworkers connected each well of a 96-well plate to an individual microfluidic channel with manually inserted ESI needles to avoid cross-contamination.[169] Each well could be individually pressurised with nitrogen gas to induce flow in the microfluidic channels. The chip was positioned on a computer-controlled translational stage in front of a mass spectrometer. Flow through one microfluidic channel at a time was induced. By moving the chip with the stage, other reservoirs could be sequentially pressurised to induce a flow through the corresponding microfluidic channel and electrospray emitter. A stationary high-voltage electrode was positioned such that during the movement of the translational stage, the high voltage was connected only to the pressurised channel. The electrodes were made of conductive epoxy. The reservoirs were alternately loaded with 5 μL of angiotensin II and angiotensin III solutions, and the contents of each reservoir was analysed by ESI-MS. Fig. 7 shows the 96 spectra obtained. Clearly, sample cross-contamination did not occur. However, several channels were blocked during the manual insertion and gluing of the 96 ESI needles, as evidenced by the absence of peaks in some of the mass spectra in Fig. 7. In a later contribution from the same group, a more stable interface was developed, in which a 96-well plate was connected to a glass chip enabling CE separation and electrospray ionisation-MS detection of the separated analytes.[39] The main difference with the previous publication is that the 96 microfluidic channels shared a single electrospray needle. Moreover, the new chip has CE separation capability. The sample is introduced into a double-T injector using a combination of vacuum and high pressure, after which the sample is electrokinetically injected onto a separation channel prior to mass spectrometric analysis. Note that sample loading into a double-T injector is normally performed with electrokinetic flow. Consecutive injections of the tryptic digests of six different proteins with thorough intermediate washing steps gave mass spectra without observed sample carry-over. The separation performance was comparable to that of a separation performed in a fused-silica capillary of the same length.

In a different high-throughput application, Tan and coworkers presented the fabrication of 8 parallel, solid-phase extraction (SPE) columns made in Zeonor (a cyclic olefin) by hot-embossing.[120] The advantage of using parallel SPE columns is that cross-contamination between samples is avoided and analysis can be performed with a higher throughput. SPE material was photo-polymerised inside the microfluidic channels (butyl methacrylate/ethylene dimethacrylate) at a predefined location. Fluidic connections were made using 360 μm od fused-silica capillaries. As a test, 10 μL of human urine was spiked with imipramine, a common antidepressant (0.025 μg mL^{-1}–10 μg mL^{-1}). The imipramine was successfully trapped and eluted with acetonitrile. A P450 metabolism study involving human liver microsomes was performed in a similar way, with the SPE column being used for the preconcentration and clean-up of the metabolites. A sample containing the microsomes was spiked with imipramine, and metabolic products of the imipramine were detected after incubation. Other examples of chips on which high-throughput analysis can be performed are described in the previous section. These were performed in PDMS[103–105] and silicon[79] devices. In an alternative approach, Li *et al.* interfaced an autosampler with a single separation-channel chip for rapid, sequential analysis of samples by microchip electrophoresis followed by detection using nanoESI-MS. Up to

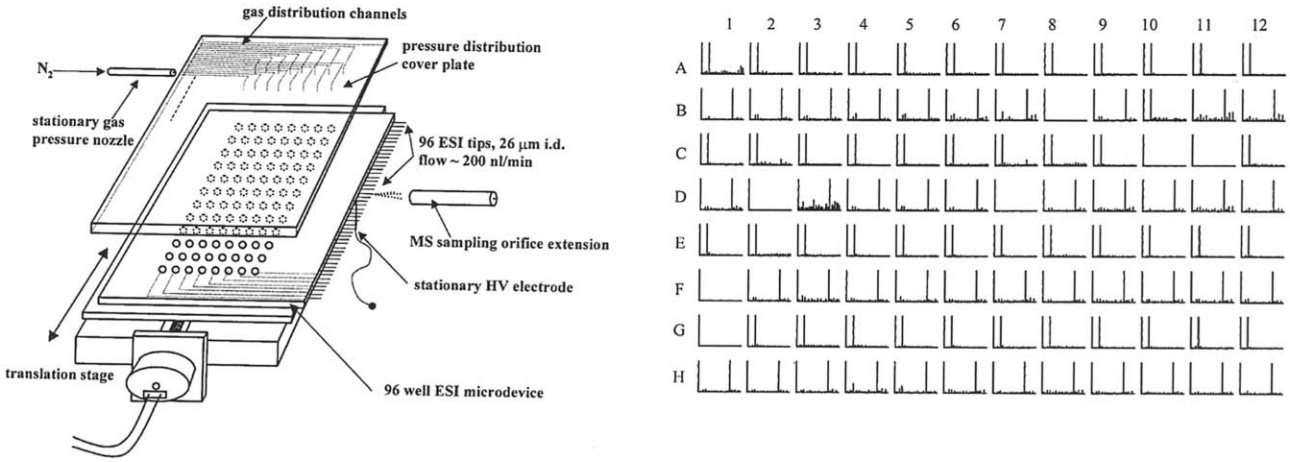

Fig. 7 A 96-well plate is shown on the left-hand side, with each individual well coupled to a microfluidic channel and electrospray emitter. On the right-hand side of the figure, 96 mass spectra obtained from the 96 wells are presented. Five microlitres of solutions of angiotensin II and angiotensin III (10 μg mL^{-1}) were alternately loaded into the sample wells.[169] [Reprinted with permission from ref. 169. Copyright 2000 American Chemical Society.]

30 samples of trace-level tryptic digests could be analysed per hour, with less that 3% sample carry-over.[49]

Examples of sample pretreatment and separation

Several types of analytical function have been integrated on-chip, of which a few have already been discussed in the previous sections. Table 3 and Table 4 present a relatively complete overview of all the various analytical separations and sample pretreatment functions, including sample clean-up and preconcentration, that have been performed on microfluidic devices coupled to ESI-MS. In Table 3, a distinction is made between sample pretreatment performed on- or off-chip. On-chip sample pretreatment indicates that all analytical functions were integrated on the chip. Off-chip pretreatment is used to refer to systems in which some of the sample handling steps were performed off-chip (*e.g.* the use of an external trap column) but coupled on-line to a microfluidic device for further processing. These systems are listed in Table 3 according to the off-chip procedures performed in each case. A few examples dealing with separations and pretreatment are outlined in the next sections.

Separations performed on-chip

Zhang *et al.* describe the capillary electrophoresis (CE) separation of peptides, proteins and tryptic digests in a glass chip coupled to ESI-MS using a novel pressure-assisted sample introduction scheme.[38] Electrical contact for ESI was made through a buffer reservoir and a liquid junction at the end of the separation channel. Injection could be performed electrokinetically or by using vacuum to draw the sample into the injection region using a modified set-up. An ESI needle was inserted into a 400 μm-deep channel etched at the end of the separation channel. The inlet of the needle was positioned approximately 50 μm from the exit of the separation channel.

Table 3 Separation mechanisms integrated on-chip and coupled with ESI to MS

Separation	Sample type	References
CE	Amino acids	63,157
	Peptides	38,40,44,52,56,71, 109,114,157
	Proteins	38,158
	Digested proteins	38–40,43,44,47,49–52, 71,112,159
	SDM[a]	44,61,62,118,127,157
	SDM[a] human plasma	61,62
	Glycoproteins	48
	Membrane proteins	48
	Dye	55
IEF[b]	Proteins	130
Frontal analysis	Digest proteins	57
CEC[c]	Digested proteins	53
RP HPLC	Digested proteins	25,99,131,135,183
	Plasma proteins	135
	Phosphoproteins	184
Graphitized carbon chromatography	Oligosaccharides	136
2-D (SCX/C18) LC[d]	Digested proteins and plasma proteins	135

[a] Small drug molecules. [b] Isoelectric focusing. [c] Capillary electrochromatography. [d] 2-D ion exchange/C18 liquid chromatography.

Table 4 Sample pretreatment steps which have been demonstrated either on-chip or off-chip but directly coupled on-line to a microfluidic device

Sample pretreatment	Sample	References
On-chip		
Desalting/clean-up		
Microdialysis	Oligonucleotide	128
	Proteins	128
Dual microdialysis	Mixture of proteins	129
	Cell lysate	129
SPE[a]	SDM	116
	SDM in urine	120,124
	Peptides	114
	Digested proteins	131,183
Membrane SPE	Proteins, peptides, SDM	143,144
FFE[b]	Proteins	155
H-filter[c]	Proteins	164
Preconcentration		
Membrane SPE	Proteins, peptides, SDM	143,144
SPE	Digested proteins	51,131,183
	SDM in urine	124
Sample stacking	Digested proteins	47
ITP[d]	Peptides	40,112
Ultrafiltration	SDM	126
Affinity dialysis	Aflatoxins	126
Immunoaffinity	Digested proteins	51
Pretreatment		
Tryptic digestion	Proteins	42,43,54,112,161
Electrochemistry		
3-electrode mode	Neurotransmitters	115
Off-chip		
Desalting/clean-up		
Enzymatic digestion	Proteins	159
Desalting	Digested proteins	159
SPE	Digested proteins	47,57
	Enzymatic reaction SDM	116
Clean-up and preconcentration		
Ion-exchange (SCX)	Digested proteins	135
	Plasma proteins	135
SPE	Enzymatic reaction SDM	116

[a] Solid-phase extraction. [b] Free-flow electrophoresis. [c] Filtering by diffusion. [d] Isotachophoresis.

Surrounding this gap, a liquid junction with background electrolyte was used to decouple the CE current from the ESI capillary. Eluting compounds from the separation channel were hydrodynamically focused in the ESI needle and measured with MS. Linear polyacrylamide or PVA was used as a coating on the channels to minimise adsorption of sample components.

Lazar *et al.* made a glass chip for CEC analysis of protein digests.[53] The stationary phase was a monolithic column made by photopolymerisation of glycidyl methacrylate/methyl methacrylate/ethylene glycol dimethacrylate polymer inside the microfluidic channels. After polymerisation, the monolith was positively charged by coating with *N*-ethylbutyl amine. A separation potential was applied over the monolith between two reservoirs. The reservoir near the end of the channel, *i.e.* the electrospray emitter, was separated from the separation channel by a porous glass disc that allowed for the exchange of ions but not bulk flow. Dilution effects in the separation channel were avoided in this manner. A peptide sample was successfully separated on a positively charged monolithic phase and measured with electrospray ionisation using an inserted fused-silica capillary.

On-chip enzymatic digestion and separation

Wang and coworkers presented a chip with an integrated bed of 40–60 μm beads on which the enzyme trypsin was immobilised (see Fig. 8).[43] Samples containing proteins were flushed through this bed at a flow rate of 0.5–60 μL min^{-1}. Separation of the peptides was performed on the same chip using CE followed by ESI through an inserted fused-silica needle. For melittin, a residence time of only 5 s in the enzymatic bed was enough for full conversion (this took 10 to 15 min with conventional, solution-phase tryptic digestion experiments). The digestion was also performed with bovine serum albumin (BSA) and was completed at a flow rate between 0.5 and 1 μL min^{-1}, corresponding to a digestion time of 3–6 min. An interesting aspect of this device was that digestion times could be precisely controlled and simply varied by altering the flow rate, and thus the residence time, of sample in the trypsin reactor. The detection limit for digestion of cytochrome C was 2 μM or 12 picomole (only 2 μL of sample was required).

Gao *et al.* presented a microfluidic device for on-line protein digestion followed by ESI-MS analysis or by transient capillary isotachophoresis/CZE ESI-MS.[112] Trypsin was immobilised on a poly(vinylidene fluoride) membrane fixed between two microfluidic channels in two PDMS slabs. A sample containing cytochrome C was flushed across the membrane and digested in 10 min to yield complete sequence coverage. Diffusion-limited reaction kinetics in protein digestion were eliminated due to the submicron diameter of the membrane pores. The digestion time was reduced to only 36 s by reducing the concentration of cytochrome C to 10 μg mL^{-1}. Membrane digestion was at least 500–1000 times faster than solution digestion. Interfering peaks in the mass spectra originating from autolysis of the proteolytic enzyme itself were reduced, due to the enhanced digestion speed. An even further increase of the speed of digestion was observed when the temperature of the reactor was raised to 50 °C. The device could be used over a period of two weeks without loss of digestion activity. The same device has been used in the capillary isotachophoresis mode, where peptides of a cytochrome C digest were separated in less than 7 min.

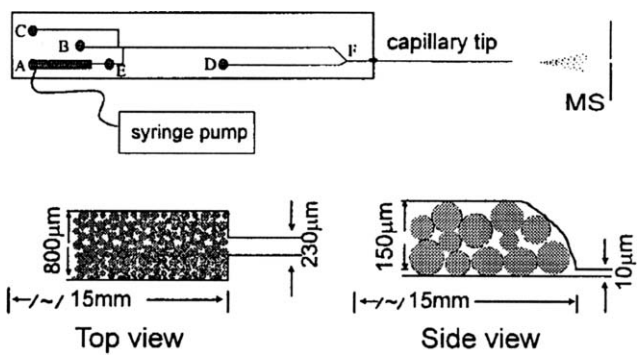

Fig. 8 Glass chip for tryptic digestion of proteins with integrated CE separation and inserted fused-silica ESI emitter. The upper figure shows the layout of the chip. Below, expanded top- and side-views of the packed trypsin bead bed is shown. The separation channel was 10 μm deep and 30 μm wide. A flat-bottomed hole was drilled in the side of the chip (200 μm) and a gold-coated nano-ESI capillary tip (185 μm od, 50 μm id) was inserted and glued. The microfluidic channels were coated with a polycationic coating to prevent protein and peptide adsorption.[43] [Reprinted with permission from ref. 43. Copyright 2000 John Wiley & Sons Limited.]

Sample preconcentration and separation

Li *et al.* have presented two approaches for sample preconcentration of gel-isolated proteins followed by injection onto a CE channel and subsequent analysis by ESI MS.[47] The first approach is by sample stacking, the second by selective adsorption of hydrophobic compounds on an off-chip preconcentrator. Narrower peaks and a 3- to 50-fold improvement in sensitivity was obtained with sample stacking, compared to the sensitivity obtained without stacking. Also, more sample could be injected onto a column. Sample preconcentration of certain peptides by SPE prior to MS analysis increased the detection limit even further. However, other peptides with low affinity for the SPE material exhibited a higher LOD. Later, the authors showed the integration of particulate material on-chip for SPE adsorption and affinity capture followed by microchip CE of target peptides in human plasma and phosphopeptides of in-gel digests, respectively.[51] This system was based on one developed earlier by Li *et al.*, described previously in the section ''High-throughput analysis'', which used an autosampler for sample introduction.[49]

Sample clean-up

Dialysis. All microfluidic implementations of dialysis processes rely on using conventional, semi-permeable membranes, which are typically integrated in a sandwich structure between two substrates structured with channel networks. Smith and coworkers used two membranes on a single chip, one with a high- and the other with a low-molecular-weight cut-off, to obtain a sample free of both cellular residues and low molecular-weight salts for MS analysis. The chip, shown in Fig. 9, consisted of three structured polycarbonate (PC) layers with a cellulose ester membrane in between each pair.[129] Channel networks were ablated into the PC using an excimer laser micromachining system. The sample is introduced through the top PC layer into the serpentine channel 1 with a flow rate between 200 and 500 nL min^{-1}. The high-molecular-weight cut-off membrane ensures that only molecules with a molecular weight of below 50 000 Da can diffuse into channel 2, while the remainder goes to a waste reservoir. A hole is drilled through the middle PC plate at the end of channel 2 to connect it with channel 3. A counter-current flow of buffer solution (10 mM ammonium acetate, 10 μL min^{-1}) runs in channel 4, which is in contact with channel 3 *via* a low-molecular-weight cut-off membrane (<8000 Da). On their way to the on-line electrospray tip, the low-molecular-weight compounds diffuse into the acceptor solvent in channel 4 and are thus removed from the sample. As a result, all compounds finally reaching the mass-spectrometer have a molecular weight between 8000 and 50 000 Da. The ESI needle is integrated with the PC chip by means of a zero-dead-volume chromatographic fitting machined into the edge of the chip and connected to the outlet channel. This low dead-volume, dual-microdialysis approach was applied to remove undesired

compounds from a cellular lysate of *E. coli*. Additionally, the high concentration of salts originally present in the sample was reduced, leading to a 20-fold increase in the signal-to-noise ratio of the mass spectrum. The detection sensitivity was sufficient to perform additional MS/MS analysis for the identification of biomarkers.[129,166,185]

A three-layer chip combining dialysis and preconcentration was realised by silicon-template imprinting in a copolyester with integrated high-molecular-weight (>80 000 Da) cut-off membranes made of poly(vinylidene fluoride) (PVDF). The sample, a reaction mixture containing an aflatoxin B_1 antibody and the aflatoxins B_1, B_2, G_1, G_2 and G_{2a}, was introduced into a device which is very similar to that presented in Fig. 9.[126] The antibody binds to the aflatoxins to form complexes with molecular weights greater than 80 000 Da in the channel where the fluid is introduced. Unbound aflatoxins are separated from the complex by dialysis through a PVDF membrane. Sample flow rates of less than 100 nL min^{-1}, corresponding to a 2 min interaction time with the membrane, were required in order to remove all non-binding low-molecular-weight molecules. For subsequent preconcentration of the complex, the outlet of the microfluidic channel is connected to another channel by a through-hole. This second microfluidic channel is again separated by a high-molecular-weight cut-off membrane, which allows evaporation of water from the sample stream into a dry-air counter flow flushed on the other side of the membrane for preconcentration. Before the sample is ionised by electrospray ionisation, the antibody/aflatoxin complex is dissociated at a microdialysis junction. A two-week repeatability experiment for the analysis of aflatoxins proved that the robustness of the system was very good with day-to-day variations of 5–10%, most likely due to the electrospray ionisation conditions. The authors report in the same paper on another device that allows microdialysis to be coupled with ultrafiltration. Their test assay involved the complexation of phenobarbital antibody with barbiturates. After introduction of the sample, containing the complex and unbound barbiturates, to the device, microdialysis was performed across a 80 000 MWCO membrane to remove unbound barbiturates. The remaining barbiturate/antibody complex was dissociated with an appropriate buffer, after which the barbiturates from the dissociated complex were ultrafiltrated across the membrane to be analysed by ESI analysis. Preconcentration factors of 50 times were measured.

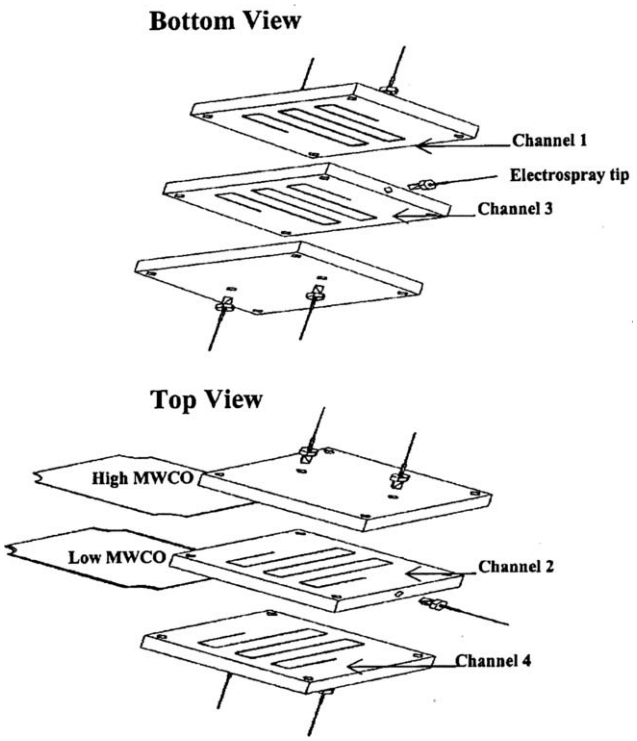

Fig. 9 Three-layer polycarbonate chip. The microfluidic channels in the three polycarbonate layers have contact with each other through high- or low-molecular-weight cut-off membranes.[129] [Reprinted with permission from ref. 129. Copyright 1999 American Chemical Society.]

Free-flow electrophoresis. Chartogne *et al.* reported a capillary isoelectric focusing (CIEF) application, in which proteins were first separated in a capillary by CIEF and then eluted into a free-flow electrophoresis (FFE) microdevice by pressure-driven flow (see Fig. 10).[155] The outlet was interfaced to the mass spectrometer *via* a coaxial sheath-flow ESI configuration. The on-chip FFE step ensured that ampholytes present in the CIEF buffer were removed. Prior to FFE, the sample is introduced in a capillary for CIEF. The device has three outlets. Ampholytes eluted from the device through one of three outlets, whereas proteins exited the device through the other two, due to the difference in electrophoretic mobility. Operation of the device was successfully demonstrated for three model proteins.

Clean-up based on diffusion. Wilson and Konermann presented a PMMA device with two inlets and two outlets that allows desalting of samples.[164] This type of device, the H-filter, is a classic example in microfluidics[186] that takes advantage of the fact that mixing between two adjacent streams occurs only by diffusion in laminar flows which are otherwise unperturbed. Since different analytes are characterised by different diffusion coefficients, analytes can be separated by virtue of the rate at which they diffuse into a receiving stream. A 1 mM ammonium acetate buffer containing cytochrome C and 25 mM sodium chloride was introduced at one inlet, while a desalting buffer (1 mM ammonium

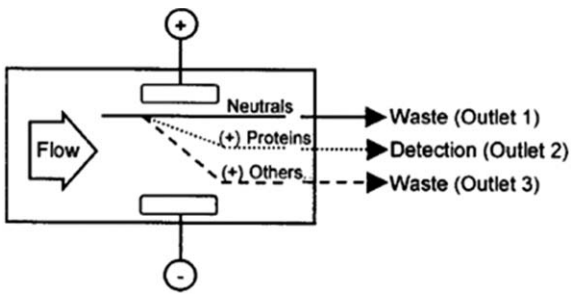

Fig. 10 Trajectories of sample components in the FFE chip. The sample, containing three model proteins, was injected into the FFE region after CIEF was performed in an external capillary.[155] [Reprinted with permission from ref. 155. Copyright 2000 John Wiley & Sons Limited.]

acetate) was introduced through the other inlet. Both streams enter the main channel and flow side-by-side, with no physical barrier such as a membrane to separate the two streams. Exchange of molecules over the interface between the two laminar flows only occurs through diffusion. Since protein molecules do not diffuse quickly, they will not diffuse to the other side of the channel on the time scale of the experiment. In contrast, sodium and chloride ions diffuse rapidly towards the other side of the channel and 90% of NaCl is removed by the desalting buffer, whereas only 30% of protein is lost. The desalted protein sample goes to the analyte outlet and is directed to the mass spectrometer. Acquired mass spectra showed significant improvement of signal-to-noise ratio upon desalting. Complete desalting using this method is thermodynamically not possible.

Sample clean-up and preconcentration. Benetton and coworkers present the kinetics of the metabolic conversion of the pharmaceutical compounds, imipramine, doxazepin and amitriptyline, into their *N*-demethylated and monohydroxylated products. This conversion was studied in a Zeonor chip containing two inlets, one for the enzyme (contained in human liver microsomes) and the other for the analyte to be digested. A porous monolithic column was integrated to perform clean-up and preconcentration before MS analysis. The kinetics of the enzymatic conversion was in good agreement with data found in the literature.[116] Sample preconcentration and clean-up of small drug molecules in urine was performed in a way similar to that described in ref. 120 using an integrated monolithic SPE column. Imipramine and enzyme were allowed to react in 25 mM Tris buffer (pH 7.4) before trapping on the SPE column. After sample preconcentration and clean-up, the trapped compounds were successfully eluted in acetonitrile containing 0.1% formic acid for ESI-MS analysis.

On-chip electrochemical labelling. Liljegren and coworkers presented a PDMS chip with three integrated gold electrodes to electrochemically oxidise dopamine prior to mass spectrometric detection. Gold-wire microcoils were integrated in the channels without making use of cleanroom facilities. A 30% conversion efficiency of the dopamine was obtained.[115] Dayon and coworkers electrochemically generated quinone mass probes on a poly(ethylene terephthalate) chip to tag cysteine moieties. The chip, made by laser ablation, had an integrated laser-ablated electrospray tip. Using this approach, up to five cysteine units could be mass-spectrometrically determined in peptides or proteins. A model for the kinetics of the tagging process is presented in a number of publications.[137,138,140,187] In another publication from the same group, the complexation of proteins with transition-metal ions was investigated. Applying a high voltage to a transition-metal electrode inserted into the reservoir served to maintain a stable electrospray. The electrode was also a source of transition-metal ions. This is due to the intrinsic electrochemical behaviour of electrospray ionisation, which leads to oxidation at the transition-metal electrode with the accompanying release of ions from its surface.[139]

On-chip reactions. A glass chip with two inlets and one outlet connected to an emitter was introduced by Brivio *et al.*[68] to perform chemical reactions on-chip coupled to mass spectrometry. The kinetics for the reaction of 4-nitro-7-piperazino-2,1,3-benzoxadiazole with several isocyanates was studied. Interestingly, the kinetics of these reactions was 3–4 times faster when performed in microchannels than under macroscale batch conditions. The authors attributed this to the shorter diffusion distances in micrometer-sized channels. Another type of chip with three inlets was used to investigate ion-suppression effects. The same group also demonstrated metal–ligand interactions, Zn-porphyrin with pyridine and related organic compounds, in a glass chip with integrated mixer. One of the interesting features of their device was the integrated nebuliser, incorporated specifically for ESI-MS applications.[70]

A glass chip with three inlets was used by de Boer *et al.*[66] for the screening of complex mixtures for the presence of bioactive molecules (see Fig. 11). The eluate from an off-chip HPLC separation was introduced onto the chip through inlet 2, together with a solution containing enzyme through inlet 3. Potentially bioactive ligands in the eluate from the HPLC column were given time to interact for 32 s with enzyme to inhibit enzyme activity, as schematically shown by eqn 1 in Fig. 11. After this interaction was complete, a substrate was introduced to the mixture through inlet 1, which monitors the remaining enzyme activity by conversion of the substrate into products. This reaction, given by eqns 2 and 3 in Fig. 11, takes place for 36 s, as defined by the volume of microreactor 5 and the flow rate. Mass spectrometric analysis as a final step gave information about the bioactivity of the ligands, in this case the inhibition of cathepsin B using protease cathepsin B as enzyme.

Spikmans and coworkers performed on-chip (glass/silicon) post-column derivatisation reactions to enhance the ionisation efficiency of primary and secondary amines with a positively charged phosphonium complex.[83] A fused-silica emitter was inserted into their chip from which electrospray for mass spectrometric analysis by a TOF-MS was performed.

Large-scale integration. Most of the examples given above describe one or two functions that have been integrated on a chip. The recent work of Xie and coworkers is outstanding as an example of sheer number of integrated components. They integrated gradient pumps, an injector, mixer, reversed-phase separation column, electrodes and ESI nozzle all into a single device.[99] The chip is made of a combination of parylene, silicon and PDMS (see Fig. 12). The mechanism used to perform pumping and injection of the sample relies on the formation of gas bubbles in enclosed cavities. Each of these pumps consists of a pair of platinum/titanium electrodes at which hydrogen and oxygen are formed due to electrolysis. An increase of the pressure in the enclosed cavities results, and is used to pump liquids through the microfluidic channels. An LC gradient of methanol/water could be generated on-chip by controlling the current in between the electrodes. The integrated pumps allow shorter cycle times for an LC run. A digested BSA sample was injected onto the reversed-phase column (1.2 cm long, 20 μm × 100 μm containing

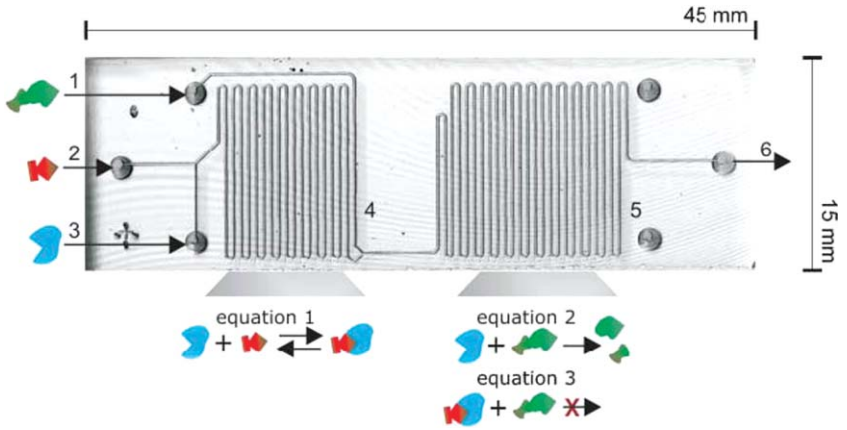

Fig. 11 Chip used by de Boer *et al.* for bioactivity screening; 1, substrate solution; 2, LC effluent; 3, enzyme solution; 4, microreactor with 1.6 μL volume; 5, microreactor with 2.4 μL volume; 6, outlet for MS detection. Enzyme hydrolyses the substrate into products (eqn 2) if no bioactive compound is eluting from the column. Bioactive compounds present in the eluate bind to the enzyme (eqn 1), resulting in a decrease of substrate turnover (eqn 3).[66] [Reprinted with permission from ref. 66. Copyright 2005 Royal Chemical Society.]

3 μm C18-modified silica particles) followed by separation and electrospray from the integrated nozzle with a 5 μm × 20 μm opening. The right-hand side of Fig. 12 shows the resulting total ion current (TIC) with an MS/MS spectrum of a separated peptide. In the lower part of the figure, the gradients generated by the integrated pumps are shown. The main drawback at the moment is that the run-to-run reproducibility is low because the initial gas/liquid volume in the enclosed cavities is difficult to control. However, the performance of the system is close to what can be achieved using state-of-the-art nanoflow HPLC systems.

Conclusions and outlook

Microfluidic chips have been coupled to mass spectrometry for a decade. The chip-ESI-MS interface has undergone a clear evolution during this time, from a more-or-less robust off-the-edge spraying approach, to inserted fused-silica needle emitters and microfabricated, fully integrated emitters. This trend is clearly demonstrated in Fig. 13, where the number of publications for the three different approaches is plotted as a function of the year of publication. Off-the-edge spraying is hardly done anymore because of the disadvantages discussed

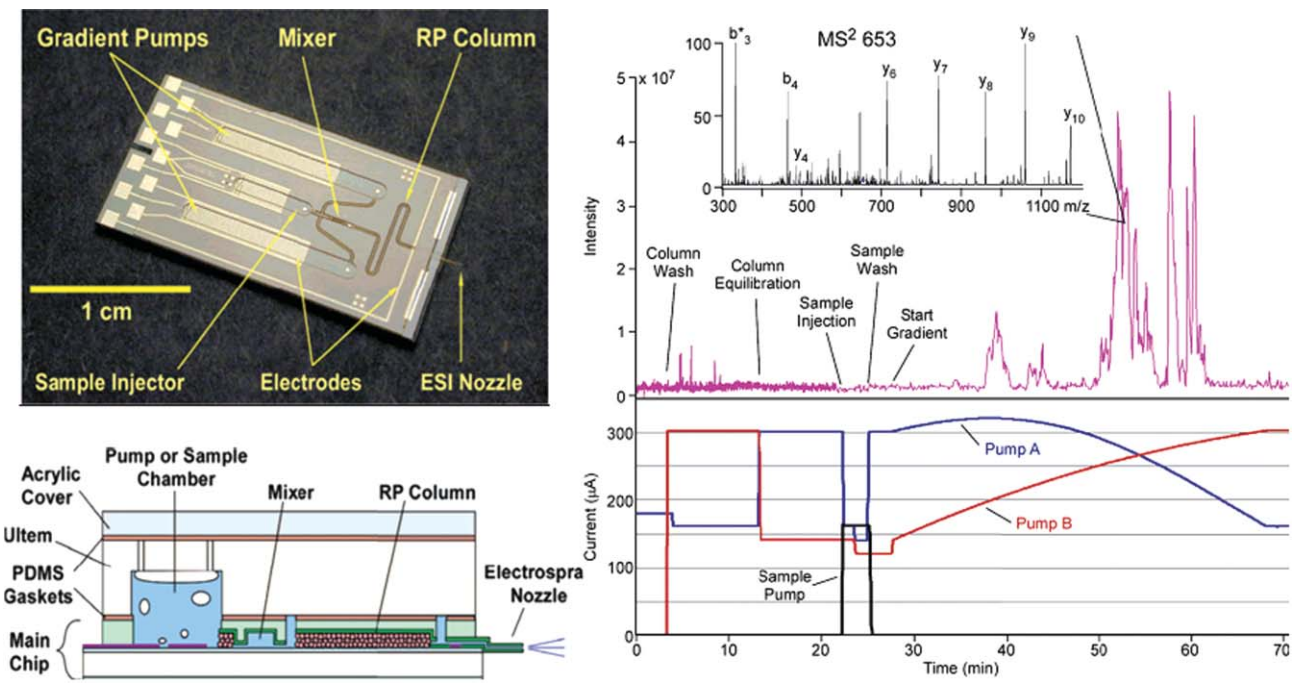

Fig. 12 Chip made of a combination of parylene, silicon and PDMS. The chip, shown on the left-hand side of the figure, has an integrated gradient pump, injector, mixer, reversed-phase column, electrodes and ESI nozzle. The right-hand side of the figure shows a chromatogram (upper panel) of a 600 fmol peptide mixture obtained from the digestion of BSA. Inset shows the MS/MS spectrum for the peptide ion with *m/z* 653.3. The panel on the lower right shows the pump current settings for the entire cycle of an LC-MS analysis of the peptide mixture.[99] [Reprinted with permission from ref. 99. Copyright 2005 American Chemical Society.]

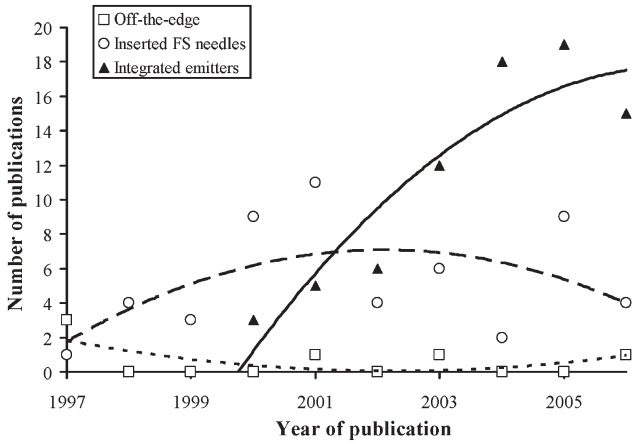

Fig. 13 Number of publications of the three different ways microfluidic electrospray chips have been coupled to mass spectrometry over the last decade. The data is fitted using second-order polynomials.

in this review. The number of sprayers employing inserted fused-silica needles is also decreasing, due in part to the manual assembly required to make these devices. This approach will in all likelihood, however, continue to exist for many more years in academia, especially in applications where dead volumes are not crucial to the application. Additionally, the technology does not require costly and/or complex cleanroom processes. For applications in the separation sciences, however, the approach is likely to die out, due to the difficulty of achieving leak-free seals and zero dead volumes between the end of the separation microchannel and the needle. In contrast, the number of publications dealing with integrated emitters has rapidly increased and is expected to continue growing. The first commercial products have appeared on the market and more are expected to follow in the near future, confirming the maturity of the field.

Most chip-ESI-MS studies deal with the same problem, namely, how to introduce a nL sample reproducibly onto a chip. Yin *et al.* found an excellent solution for this, employing an on-chip trapping column clamped in between the stator and rotor of an HPLC injection valve.[131] Using this approach, standard pumps can be used to reproducibly introduce the sample onto the chip without any losses.

A number of studies have demonstrated good analytical performance, and in several cases, better performance compared to conventional analytical techniques. Microfluidics is superior to conventional instrumentation when it comes to speed (*e.g.* fast separations), high throughput, reduced sample consumption, and the integration of multiple analytical functionality with minimal or zero dead volume between elements. Microtechnology allows the integration of sample pretreatment and clean-up steps on chip and to integrate new microfluidic phenomena that are difficult to integrate otherwise. Most publications to date have demonstrated only one or two functions integrated on a chip. However, new platforms with multiple integrated functionality are expected to be developed to a sufficient level of reliability in the near future that they will no longer just be research tools for a few experts.

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