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Research Article

A practical interface for microfluidics and nanoelectrospray mass spectrometry

We report a new method for fabricating nanospray ionization tips for MS, formed from glass substrates and the inert polymer, parylene-C. Using a single photolithography step, the emitters are formed contiguously with microchannels, such that no dead volumes are observed. In addition, because the devices are very thin (~0.3 mm) and the tips are formed at rectangular corners, the Taylor cone volumes are small, which makes the method attractive for future integration with microfluidic separations. Device performance was demonstrated by evaluating diverse analytes, ranging from synthetic polymers, to peptides, to nucleic acids. For all analytes, performance was similar to that of conventional emitters (pulled-glass capillaries and the Agilent HPLC Chip™) with the advantage of rapid, batch fabrication of identical devices.

Keywords:
Lab-on-a-chip / Mass spectrometry / Microfluidics / Nanoelectrospray ionization / Proteomics
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1 Introduction

There is wide-spread interest in coupling microfluidics to MS, with the goal of leveraging the efficiency and integration inherent in microfluidic methods with the unparalleled qualitative analytical capacity of modern mass spectrometers. This interest is heightened for applications in proteomics, as the tools currently used in this discipline fall far short of the performance needed for high-throughput analysis [1–3]. Two sample introduction methods are commonly used in MS for proteomics, ESI and MALDI. Although microfluidic devices have been integrated with MALDI [4–8] we contend that ESI, when implemented in "nanospray ionization" (NSI) mode, is the most suitable geometry for integration with microfluidics. This assertion springs from the obvious similarities between the conventional technique of interfacing LC eluent to a spectrometer by means of pulled-glass nanospray tips, and the linear geometry of microfluidic channels.

We report here the development of a new strategy for forming microfluidic–NSI devices, which builds on those reported in the literature. The microfluidic–NSI methods developed previously can be broadly classified by how the electrospray is generated, including: (i) direct spray from channels [9–14]; (ii) spray from mated, conventional tips [15–24]; and (iii) spray from microfabricated tips [25–33].

Electrospray directly from a channel (*i.e.*, the unmodified edge of a device) is the most straightforward approach for interfacing microchannels with MS [9–14]. The first microchannel–NSI interface was reported by Xue *et al.* [9], in which analyte was sprayed from the flat edge of a glass channel. The authors observed that performance was limited by eluent spreading at the interface, resulting from the nontapered geometry and the hydrophilicity of the substrate. Although some researchers have improved upon this technique by tapering the edge of a substrate [12] or by integrating hydrophobic PTFE (or Teflon) surfaces at the tip [13], the spreading of eluent is a major drawback for this technique. One creative strategy for overcoming this problem was recently reported by Bedair and Oleschuk [14], who constructed a polymeric monolith at the edge of an open channel, which resulted in stable ionization from several mini sprays generated by pores at the monolith interface.

The problems associated with direct spray from the edges of chips prompted the development of an alternative geometry for interfacing microfluidics with MS: mating microchannels to conventional pulled glass capillary tips [15–24]. These devices are capable of generating mass spectra with sensitivities similar to those of conventional techniques (~100 nM) [15, 16]. A variety of materials have been used in this device format, including glass [17, 19–21], poly(methyl methacrylate) (PMMA) [18], glassy carbon [22]

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Abbreviations: NSI, nanospray ionization; **PG,** parylene-glass; **PG-NSI,** parylene-glass NSI; **PMMA,** poly(methyl methacrylate); **TIC,** total ion count

and PDMS [23]. A critical problem for this geometry is observed when performing chemical separations: resolution is compromised as analytes pass through dead volumes in the interface between chip and capillary. It has been shown that the dead volume can be reduced by drilling substrates prior to mating with tips; however, this technique requires careful attention in the fabrication process [24]. Overall, we assert that this device geometry is not likely to be useful for the development of proteomic analysis tools requiring high-resolution separations.

A third strategy for microfluidic–nanospray interfaces, microfabricated tips, has become the most popular [25–33]. In this category, a wide range of fabrication methods have been employed. Schilling *et al.* [25] micromilled a nozzle in PMMA, and demonstrated stable spray as a function of nozzle dimensions. Xie *et al.* [26] used parylene-C to fabricate ESI tips on silicon microfluidic devices, enabling integrated LC with MS with comparable performance to conventional techniques (see also [27]). Parylene membranes on plastic substrates [28], PDMS [29], and silicon substrates [30, 31] have also been used for microfluidic NSI. Hoffmann *et al.* [32] used a heated puller to construct nanospray emitters from commercial glass devices, obtaining tips with comparable geometry to conventional, pulled capillaries. The one drawback for microfabricated NSI interfaces is that the devices typically require arduous, time-consuming cleanroom fabrication, and are thus not likely to be viable for widespread use.

Of the microfabricated NSI interfaces reported in the literature, the one developed by Yin *et al.* [33] is perhaps the most promising. This device, which has been commercialized by Agilent Technologies as the HPLC Chip™ (http://www.chem.agilent.com/Scripts/PDS.asp?lPage=38308, accessed on 08/02/2007), is formed by laser ablation of a polyimide substrate, and is capable of separations and MS with detection characteristics (peak resolution, detection limits, background ion level, *etc.*) similar to those obtained by conventional capillary-scale methods. This device is gaining popularity in the analytical community [34, 35], and is the first true competitor to pulled-glass capillary NSI tips. Unfortunately, these commercial devices are very expensive (*i.e.*, ~$500), and the price is not likely to decrease with time, as laser ablation is inherently a serial process, not well suited for batch production.

Here, we report the development of a new microfluidic–NSI device fabricated by plasma etching of parylene-C on a glass substrate. Parylene is unique relative to many polymeric materials used in microfabrication (*e.g.*, PDMS, PMMA, *etc.*), because of its very high chemical inertness – parylene is compatible with aggressive organic solvents, strong acids, and strong bases. The new devices, which we call parylene-glass NSI (PG-NSI) chips, fall into the first category of microfluidic–NSI interfaces: spray is generated from the unmodified edge of a device. Unlike the previous methods in this category [9–14], eluent spreading in the new geometry is significantly limited by using very thin substrates and by the fabrication of tips ending at the corners of the devices. Additionally, the new method requires only a single photolithography step, with no cutting or etching of glass substrates. The devices are thus very straightforward to fabricate and have comparable performance to both commercial pulled-glass emitters and the Agilent HPLC Chip. For these reasons, we believe this method has the potential to become useful for a wide range of researchers developing microfluidic–NSI methods for proteomic analyses.

2 Materials and methods

2.1 Reagents and materials

Unless otherwise indicated, reagents were from Sigma–Aldrich (Oakville, ON). Parylene-C dimer and Silane A174 were from Specialty Coating Systems (Indianapolis, IN). Hexamethyldisilazane (HMDS) was from Shin-Etsu MicroSi (Phoenix, AZ). Shipley S1811 photoresist and MF321 developer were from Rohm and Haas (Marlborough, MA), chromium was from Kurt J. Lesker Canada (Toronto, ON), and CR-4 chromium etchant was from Cyantek (Fremont, CA). Microscope slides (75 × 25 mm, 1 mm thick) and cover slips (no. 1, 0.15 mm thick) were from Fisher Scientific Canada (Ottawa, ON). Plastic transparencies printed on an outsourced printer (4000 DPI) were used as photolithographic masks.

For MS, ultramark 1621 (Thermo Fisher Scientific, Waltham, MA) was diluted in ACN according to the instructions of the manufacturer (1 μM final concentration). An HPLC standard containing methionine enkephalin, leucine enkephalin, and angiotensin II was diluted in a 1:1 methanol/deionized (DI) water solution with 0.3% acetic acid in total volume (1 μM final concentration). Unless otherwise indicated, angiotensin I and II were also diluted in the same manner (10 and 1 μM final concentration, respectively). 20-mer predesalted DNA oligonucleotide (5′AGCAGAGCGACCTCAATGAT3′) (1 μM final concentration) was dissolved in ammonium acetate buffer at pH 7.5, and diluted in 1:1 methanol/DI water. Insulin from bovine pancreas was dissolved in acetic acid according to the instructions of the manufacturer and diluted in 1:1 methanol/DI water.

2.2 Device fabrication

Glass substrates (microscope slides and cover slips) were cleaned in piranha solution (3:1 conc. sulfuric acid, 30% hydrogen peroxide) for 10 min, dried, and dip-coated with Silane A174. After drying, the substrates were coated with a layer of parylene-C (20 μm) using a vapor deposition instrument (Specialty Coating Systems) followed by coating with a sacrificial layer of chromium (300 nm) by e-beam evaporation (Edwards Auto 306, Wilmington, MA). After cleaning (acetone, methanol, and DI water), drying, and priming with HMDS, S-1811 was spin-coated onto the substrates

(5000 rpm, 30 s). After soft-baking (2 min, 104°C), substrates were photolithographically patterned by exposure to UV radiation (365 nm, 35 mW/cm^2, 30 s) using a Karl-Süss MA6 mask aligner (Garching, Germany). Note that the conditions used in this process – 5000 RPM spinning velocity, and 30 s UV exposure – were necessary to minimize the formation of beads of photoresist on the edges of the substrates, which interfere with the formation of tips.

After developing in MF321 (~30 s), the exposed chromium was etched in CR-4 (~30 s), and then the exposed parylene was etched by reactive ion etching (RIE, 150 W, 98 sccm O_2, 200 mTorr for 15 min) in a Trion Phantom etcher (Clearwater, FL). The remaining chromium was then etched away and the devices dried. An additional layer of parylene (2 μm) was deposited onto the substrates to make all surfaces uniformly hydrophobic. A second set of substrates was also coated with parylene (5 μm), and then bonded to the patterned substrates in a vacuum oven (200°C, 48 h) while held together by a vise (~20 MPa) [36]. With exception of the bonding, the described steps required ~48 h *per* batch of 20 devices and were conducted in a class 100 cleanroom at the Emerging Communications Technology Institute (ECTI) in the University of Toronto.

As shown schematically in Fig. 1a, the assembled devices had a channel leading from an inlet reservoir to an outlet tip at the corner of the device (see pictures in Figs. 1b and 1c). Typically, the channels were 20 μm deep, with widths of 300 μm tapering to 60 μm at the tips. The cross-sectional area of the tips was ~1200 μm^2. NanoPort connections (Upchurch Scientific, Oak Harbor, WA) were attached to the device reservoirs and connected to a syringe pump *via* a fused-silica transfer line (360 μm od, 100 μm id, Polymicro, Phoenix, AZ). A 1:1 mixture of methanol and DI water was flowed through the channel for 15 min to clear any impurities or particulates left in the channel and tip during the fabrication. Devices were imaged using a CCD camera and a Hitachi S-5200 electron microscope (Hitachi High Technologies America, Pleasanton, CA).

To evaluate nanospray shape, potentials (3–4.5 kV, relative to a ground electrode ~2 mm distant from the tip) were applied *via* a conductive union (Upchurch Scientific). Pictures were collected *via* a camera positioned horizontally relative to the devices. The volumes of the Taylor cones were estimated by extrapolating a symmetric cone from the dimensions in the pictures.

2.3 MS

The performance of home-built PG devices was compared to that of conventional pulled-capillary tips (FS360-50-30-N-20-CT 360 μm od capillary, 30 μm id tip, New Objective, Woburn, MA) and commercially available polyimide microfluidic chips (G4240-61002, 15 μm id tip, Agilent, Waldbronn, Germany; this chip is not packed with chromatographic media). Each type of tip was interfaced with an LTQ Linear IT Mass Spectrometer (Thermo Scientific, Waltham, MA), and nanoelectrospray performance (spray stability, total ion count (TIC), and sensitivity) was evaluated for several analytes, as described below. Unless otherwise indicated, analytes were flowed at 500 nL/min, with an applied potential of 3.5 kV and transfer capillary temperature of 250°C. Capillary voltages (ranging from 22 to 50 V) and other parameters were varied for each experiment to optimize the observed signal. The spectra presented were obtained by averaging 10–50 acquisitions (at a rate of 1–6 acquisitions/s), and are representative of separate analyses *per* experimental condition. The MS/MS analysis of angiotensin I was performed at 3.3 kV spray voltage; 49 V capillary voltage; 170°C capillary temperature; 120 V tube lens voltage and collision energy equal to 25%.

3 Results and discussion

3.1 Rapid fabrication

The single-photolithography-step fabrication technique described here is much faster than conventional methods, enabling the production of >20 identical devices in less than 48 h of work. We contrast this to the many elegant but highly complex methods reported previously, requiring up to three separate photolithography steps or other labor-intensive procedures to form operable microfluidic–NSI devices [25–27, 30]. The requirement of using multiple photomasks in these traditional microelectromechanical systems (MEMS) devices slows production considerably, as alignment of each new mask to the existing device patterns is tedious and time-consuming. The new method reported here is also unique in that it is likely the first microfluidic–NSI interface fabrication procedure to use printed transparencies as photomasks, enabling fast and inexpensive fabrication relative to traditional chromium-on-quartz masks. A third novelty (which

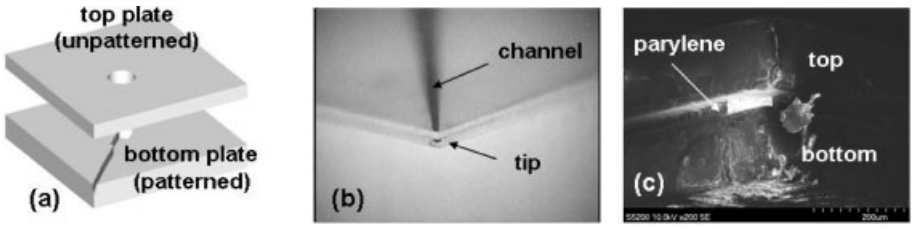

Figure 1. (a) Schematic of the fabricated device showing top and bottom plates prior to bonding. (b) Picture of the channel and tip of a device constructed from cover-slips. (c) SEM picture of the tip.

also contributes to the efficiency of the new method) is the use of preformed, inexpensive substrates (microscope cover slips), which obviates the requirement of high-precision wafer dicing to release/expose the tips [26, 27, 31].

While the new PG-NSI method compares favorably to previously reported microfabrication procedures, we note that it is slower than forming conventional, pulled-capillary emitters (which can be formed in minutes). However, integration of conventional emitters to microfluidic devices [15–23] often leads to the presence of dead volumes, which adversely affects separations. In the new method described here, the nanospray nozzle is formed contiguously with the microchannels regardless of the complexity of their design. Thus, we assert that the new method represents a significant advance, allowing for rapid interfacing of devices with a mass spectrometer without the generation of dead volumes.

3.2 Spray performance

As described in the introduction, the new method reported here falls into the first category of microfluidic–NSI devices: spray from the unmodified edges of microfluidic chips. A critical drawback for devices of this type that has been reported previously [9–13] is the phenomenon of eluent spreading at the opening of the tips. This is a significant problem for analyses requiring chemical separations, and is caused by the increased surface area (*i.e.*, the sides and edges of the tip) that is available for wetting. This phenomenon can be minimized on microfluidic–NSI tips by using complex, multistep fabrication techniques to form a tip with reduced outer diameter [25, 30], or by coating the emitter with a hydrophobic surface such as a fluoropolymer [13, 25]. Both measures require additional fabrication time, and coatings typically degrade after a few uses. In contrast, the PG-NSI devices reported here circumvent the wetting problem by (i) the use of thin substrates (when assembled, the device is ~300 μm thick), (ii) aligning the tip to the corner of the device, and (iii) the use of a hydrophobic substrate (rather than a temporary coating). When taken together, these factors result in a method with significantly reduced wetting, and small, stable Taylor cones. In short, the new fabrication method is not only rapid and straightforward, but also is well-suited for electrospray performance.

To evaluate the amount of eluent spreading at emitter tips, we captured images of sprays of a 1:1 methanol/DI water solution in the PG-NSI devices formed from microscope cover slips as well as from microscope slides (the latter have comparable thickness to devices reported previously [10, 13]). Potentials were applied between the devices and a ground electrode ~2 mm distant from the tip, and were varied until stable Taylor cones were formed. As shown in Fig. 2, the sprays formed at the two kinds of tips are quite different, originating from Taylor cones of approximately 2 nL for the thin substrates and at least 20 nL for the thick devices. The former volume is compatible with the peak volumes for microchip separations [1], making the thin devices well

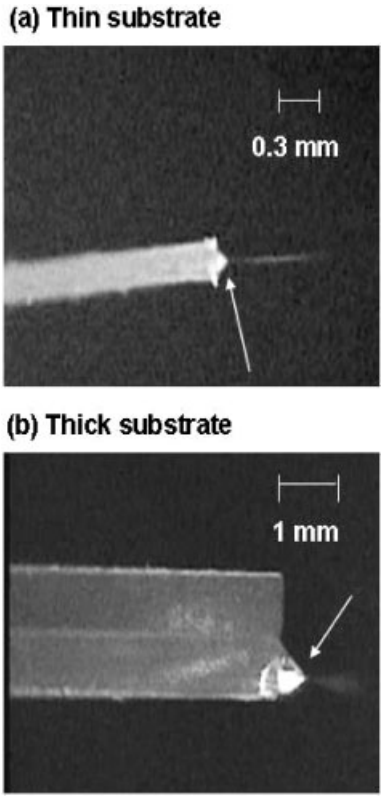

Figure 2. Taylor cones (indicated by arrows) produced by devices formed from cover slips (a) and glass slides (b). The volume of the Taylor cones on the two devices were ~2 and ~20 nL, respectively. The reduced area for wetting in (a) contributes to the formation of a small and stable Taylor cone with minimal wetting at the tip.

suited for this application. In addition, when varying the applied voltage and distance, we observed thicker devices to be more prone to corona discharges [37], leading to wetting and spray instability. It is clear that the thin devices (*i.e.*, formed from microscope cover slips) are characterized by superior spray (see Fig. 2a) and negligible dead volumes, and thus these devices were used for the remainder of the work presented here.

To characterize the stability of sprays formed at the edge of PG-NSI devices, we interfaced them to a Thermo LTQ mass spectrometer and evaluated the TIC. As shown in Fig. 3, the sprays generated by the PG-NSI devices were observed to be stable, with TICs comparable to those of a pulled glass capillary and an Agilent polyimide tip. In addition, Fig. 3 shows spectra of angiotensin II generated by each type of emitter – no "background" peaks were observed to correlate with parylene devices, which is not surprising, given its very high chemical inertness.

Several PG-NSI devices were evaluated in the course of multiple experiments – typically, they had lifetimes of ~1 week of intermittent use. The lifetime of PG-NSI tips, like those of conventional pulled-glass emitters, is limited by

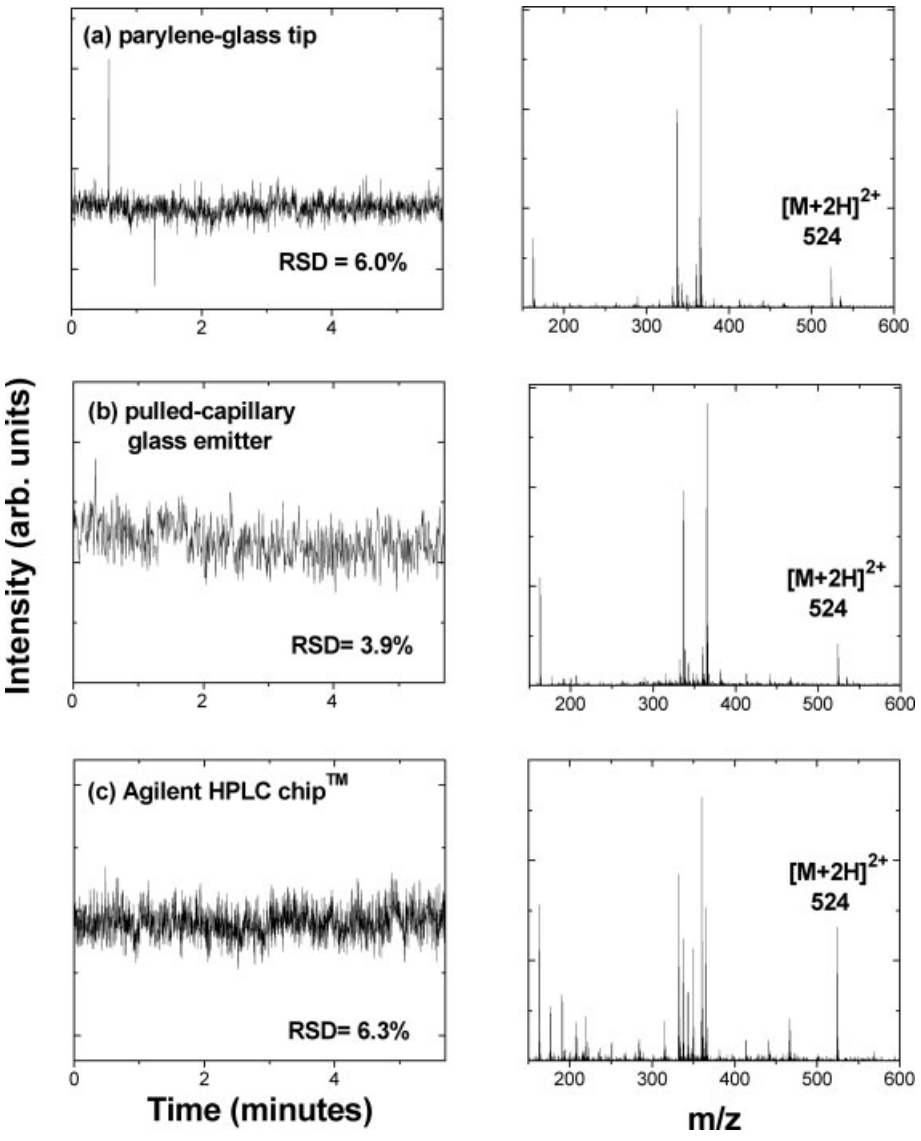

Figure 3. Left: Typical TIC traces for a PG device (a), a pulled-capillary glass emitter (b), and an Agilent chip (c). In each case, the infused solution was angiotensin II (1 μM in 50:50 methanol/DI water with 0.1% acetic acid). The applied potentials and flow rates for (a–c) were 3.4 kV/0.6 μL/min, 1.7 kV/0.5 μL/min, and 1.4 kV/0.5 μL/min, respectively. Capillary temperature was 170°C for all cases. Right: Spectra obtained for the three emitters, showing the doubly protonated angiotensin II peak (524 *m/z*).

the adsorption of analytes and other chemical constituents to the tip openings, which degrades the quality of spray. Unlike conventional emitters, however, PG-NSI tips could be recoated with 2–5 μm of parylene (by vapor deposition) and used again – recoated tips were found to have identical performance to first-generation devices. This capacity to regenerate tips is an advantage of PG-NSI devices relative to conventional emitters.

3.3 MS performance

The capacity of the PG-NSI devices to infuse samples into the MS for analysis was evaluated for several analytes ranging from synthetic polymers to peptides and nucleic acids. A spectrum of calibration standard, ultramark 1621, is shown in Fig. 4a. Ultramark, a mixture of fluorinated phosphazenes, is characterized by a series of intense singly charged peaks equally spaced by *m/z* 100 and is thus a particularly good benchmark for MS emitters [38]. A spectrum of several peptides used as HPLC standards, angiotensin II (*m/z* 1046), leucine enkephalin (*m/z* 556), and methionine enkephalin (*m/z* 574), is shown in Fig. 4b. Singly charged angiotensin II was also observed, shown in Fig. 4c. A spectrum of a 20-mer DNA oligonucleotide is shown in Fig. 4d – this is of note because analytes carrying large numbers of negative charge in solution phase (such as DNA) are not typically analyzed by positive mode MS (and thus are typically only observed when using well optimized NSI-MS systems). In all cases, the analytes could be identified.

In the past decade, MS/MS combined with CID for peptide sequencing has become a method of choice for proteome profiling [1–3]. To demonstrate the compatibility of

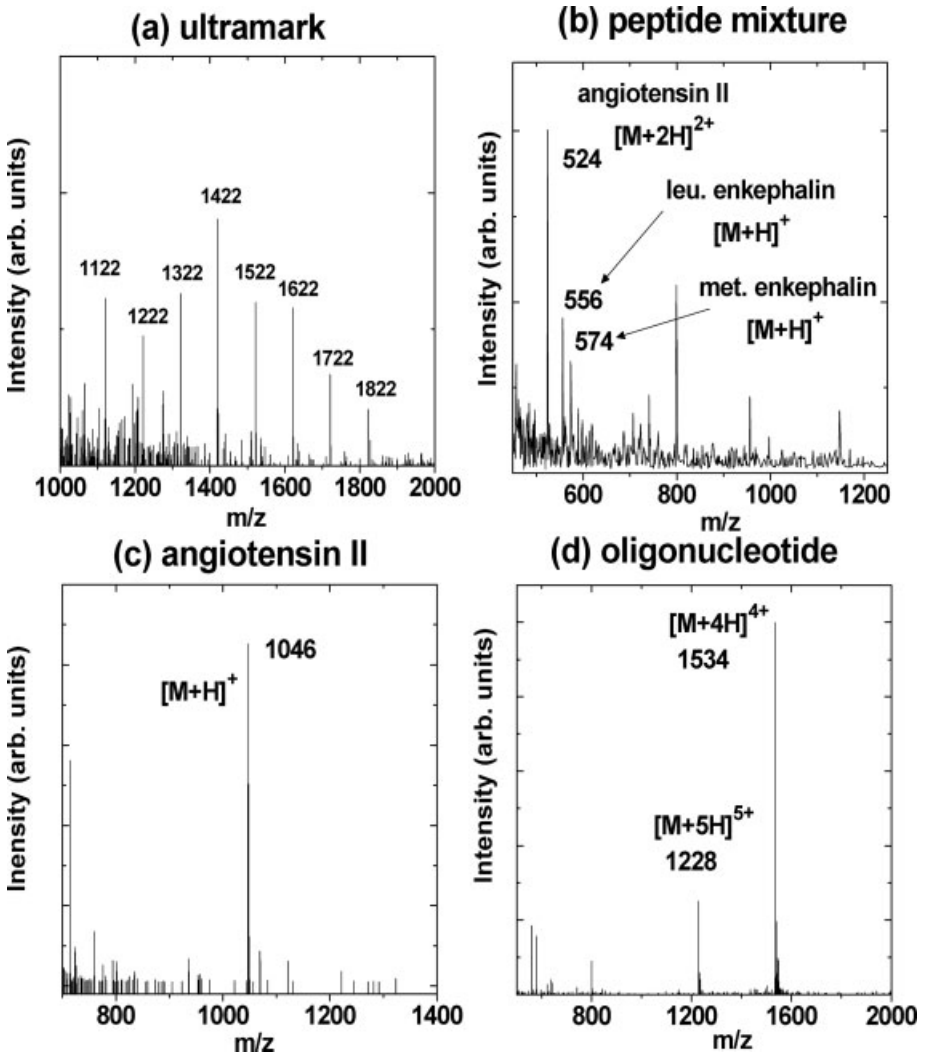

Figure 4. Mass spectra generated using PG-NSI devices. (a) Ultramark calibration standard (1 μM); (b) HPLC peptide standards (1 μM), including singly protonated leucine enkephalin (556 *m/z*), methionine enkephalin (574 *m/z*), and doubly protonated angiotensin II (524 *m/z*); (c) singly protonated angiotensin II (1 μM) (1046 *m/z*); (d) 20-mer DNA oligonucleotide (1 μM), $[M + 5H]^{5+}$ (1228 *m/z*) and $[M + 4H]^{4+}$ (1534 *m/z*).

the new method with peptide sequencing, a PG-NSI device was used to analyze angiotensin I by MS/MS. As shown in Fig. 5a, multiple parent ions were observed in the first mass selection (Fig. 5a), and after isolation of the triply protonated ion and CID, several b- and y-ion peptide fragments were identified in the second mass selection, as shown in Fig. 5b.

Finally, to characterize the detection limits of the new device, we analyzed standard solutions of insulin. As shown in Fig. 6, a PG-NSI device can detect 100 and 10 nM concentrations, with good S/N. We estimate the detection limit to be ~2 nM (for S/N of 3). This is comparable to the detection limits of pulled-capillary (data not shown) and polyimide microfluidic emitter devices (~0.5 nM, S/N ~ 3). In short, the new PG-NSI devices are similar to conventional techniques, with the advantage of rapid, batch fabrication of emitter tips with identical geometries.

4 Concluding remarks

We present a new method for the fabrication of NSI tips for interfacing microfluidic devices to mass spectrometers (PG-NSI devices). The construction of these tips is relatively simple, as the spray is generated from the unmodified edge of a device, not requiring dicing of substrates to release/expose the tips. However, in contrast to the previous methods of this type that have been reported, eluent spreading in the new geometry is significantly limited by (i) the use of thin substrates (when assembled, the device is ~300 μm thick), (ii) aligning the tip to the corner of the device, and (iii) the use of a hydrophobic substrate (rather than a temporary coating).

When analytes are sprayed from the new tips, small Taylor cone volumes are observed, which makes the method attractive for the integration with microfluidic separations. The devices were evaluated for several analytes, within a con-

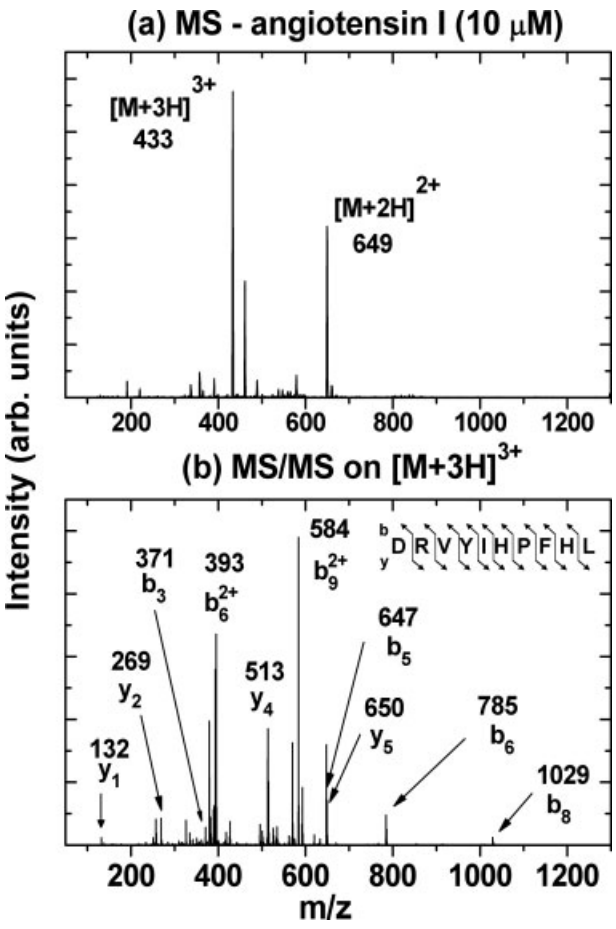

Figure 5. (a) Spectra of angiotensin I (1296 *m/z*) (10 μM) obtained using a PG device, emphasizing the doubly (649 *m/z*) and triply (433 *m/z*) protonated parent ion peaks. (b) MS/MS performed on the 433 *m/z* parent ion. Several peaks were identified as b- and y-ion peptides (see inset on top right).

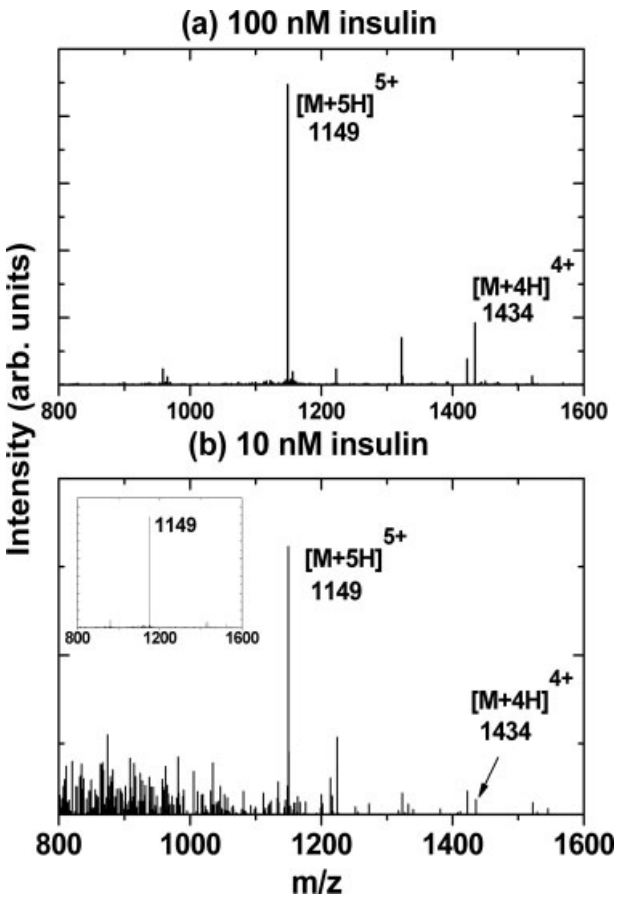

Figure 6. Evaluation of PG devices for the detection limit of insulin. The peaks at 1149 *m/z* ($[M + 5H]^{5+}$) and 1434 *m/z* ($[M + 4H]^{4+}$) are observed at both 100 nM (S/N ~ 200) (a) and 10 nM (S/N ~ 17) (b) concentrations (the spectrum shown in (b) is an average of only two acquisitions). The inset shows a spectrum acquired using the Agilent chip (S/N ~ 160).

centration range of 10 nM to 10 μM. In all cases, the spectra were comparable to those collected using conventional pulled-glass capillaries and the Agilent HPLC Chip. In ongoing work, we are building devices with more complex channel geometries, to effect sample injections and separations integrated with the NSI emitters described here, aiming at lab-on-a-chip applications for proteomics analyses.

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