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Ultrasensitive Nanoelectrospray Ionization-Mass Spectrometry using Poly(dimethylsiloxane) Microchips with Monolithically Integrated Emitters

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Summary

Poly(dimethylsiloxane) (PDMS) is a widely used substrate for microfluidic devices, as it enables facile fabrication and has other distinctive properties. However, for applications requiring highly sensitive nanoelectrospray ionization mass spectrometry (nanoESI-MS) detection, the use of PDMS microdevices has been hindered by a large chemical background in the mass spectra that originates from the leaching of uncross-linked oligomers and other contaminants from the substrate. A more general challenge is that microfluidic devices containing monolithically integrated electrospray emitters are frequently unable to operate stably in the nanoflow regime where the best sensitivity is achieved. In this report, we extracted the contaminants from PDMS substrates using a series of solvents, eliminating the background observed when untreated PDMS microchips are used for nanoESI-MS, such that peptides at concentrations of 1 nM were readily detected. Optimization of the integrated emitter geometry enabled stable operation at flow rates as low as 10 nL/min.

Introduction

Microfluidic devices are expected to play a growing role in proteomics, metabolomics and other biochemical analyses due to their ability to integrate multiple sample handling and separation steps, and to manipulate small sample volumes in an automated fashion.^{1–5} Lab-on-a-chip approaches will be especially important for small samples (*e.g.*, single cells and limited cellular populations) not effectively processed using conventional methods.^{6–8} While a variety of detection strategies have been employed for microfluidic analyses,⁹ the most widely used method is laser-induced fluorescence (LIF), which provides high sensitivity and is easily implemented for planar, optically transparent devices. However, sample labeling is typically required and identification of large numbers of proteins or peptides is not feasible using LIF. In contrast, mass spectrometry (MS) provides information-rich detection for proteomics with the ability to identify large numbers of analytes as well as provide sequence and structural information.^{10–11} Nanoelectrospray ionization (nanoESI) is favored for the interfacing of microfluidic devices with MS detection due to its high sensitivity, which increases at lower flow rates, and other benefits that include improved quantitation and reduced ionization suppression and bias effects.^{12–14}

While a large number of methods have been developed to couple microchips with ESI-MS (see Refs [15, 16] for reviews), few designs have demonstrated robust operation at the low flow rates (*e.g.*, <50 nL/min) required to fully realize the benefits of nanoESI.¹⁴ Indeed, the high aspect ratio and narrow orifice of tapered fused silica capillary nanoESI emitters have proven difficult

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to replicate in planar microfluidic systems. As such, fused silica capillaries with tapered emitters have been inserted into microchips and have successfully operated at flow rates as low as 20 nL/min.^{17–19} However, the significant amount of manual effort required, and the challenge of achieving a low dead volume connection have driven the development of microfluidic devices with integrated nanoESI emitters. Licklider et al.²⁰ developed a micromachining procedure to incorporate parylene electrospray emitters in silicon microchips that could operate at ~50 nL/min. Wang et al.²¹ incorporated porous hydrophobic membranes at the terminus of polymer microchips. Mass spectra were shown for 100 nL/min flow rates, and electrospray current measurements were made at flows as low as 10 nL/min. Hoffman et al.^{22,23} machined a narrow cylinder at the terminus of a glass microchip electrophoresis (ME) channel, which could then be tapered to a fine point as with conventional capillary emitters; stable electrospray was demonstrated at flow rates of 25 nL/min. Mellors et al.²⁴ recently achieved stable electrospray operation at 40 nL/min for MS analysis using a glass ME device by spraying from the corner of thin substrates. These advances, and ongoing efforts to further simplify nanoESI source fabrication or adapt to alternative devices substrates will make high sensitivity MS detection more accessible for chip-based analyses.

Our group previously developed a robust integrated emitter for PDMS microchips that enables broad stability in the cone-jet mode at flow rates as low as 50 nL/min.²⁵ The electric field enhancing taper was formed by simply making two vertical cuts with a razor blade such that the channel terminated at a sharp corner, while the naturally hydrophobic PDMS surface helped to maintain a small, well defined Taylor cone.²⁶ Analyte was delivered through a microchannel while the high voltage for electrospray was supplied through electrolyte in a closely spaced parallel channel. Electrical contact was thus provided in the Taylor cone itself where the channels terminated, forming a liquid junction without sample loss or dilution. This approach has also been combined with droplet-based microdevices that extracted subnanoliter oil-encapsulated droplets to an aqueous stream for analysis by nanoESI-MS with minimal dilution.

A challenge encountered in our research and also reported by others^{27–29} using PDMS microdevices for ESI-MS is the presence of uncross-linked species comprising low molecular weight (LMW) oligomers and other contaminants that can leach from the PDMS bulk and dissolve in the organic/aqueous co-solvents used for ESI-MS. These contaminants contribute to chemical noise in the mass spectra and limit achievable sensitivity. To suppress this background interference, researchers have allowed PDMS microchips to cure for extended times (as long as 48–72 h^{27–29}), which enhances cross-linking reactions. This process is time-consuming, and some uncross-linked species remain.

In the present work, we have improved the coupling of PDMS microchips with nanoESI-MS detection by applying a solvent-based extraction procedure to device substrates prior to bonding, eliminating the chemical background species that otherwise originate from the substrate. We also optimized the emitter geometry, enabling stable operation of the devices at flow rates as low as 10 nL/min. The optimization yielded sub-nanomolar concentration detection limits for MS-only analyses (*i.e.*, without employing MSⁿ to further reduce chemical background) and mass detection limits of ~40 zmol based on the amount of sample consumed to generate a spectrum, the best figures of merit achieved to date for microfluidic devices with monolithically integrated nanoelectrospray emitters.

Experimental

Materials

HPLC-grade methanol and acetone were purchased from Fisher Scientific (Fair Lawn, NJ). Diisopropylamine (>99.5%), toluene, glacial acetic acid, reserpine, porcine angiotensinogen

1-14, angiotensin I, leucine enkephalin, methionine enkephalin, syntide 2 and fibrinopeptide A were obtained from Sigma-Aldrich (St. Louis, MO). Water was purified using a Barnstead Nanopure Infinity system (Dubuque, IA). PDMS elastomer base and curing agent were purchased as Sylgard 184 from Dow Corning (Midland, MI). The solvent for ESI was prepared by mixing water and methanol in a 9:1 (v/v) ratio and adding 0.1% (v/v) acetic acid. All tested samples, including reserpine and peptides, were prepared in the electrospray solvent.

PDMS microchip fabrication

The PDMS microchips were fabricated using well established soft lithography techniques.²⁵ First, an SU-8 photoresist (Microchem, Newton, MA) mold was produced using standard photolithographic patterning from a photomask that was designed using IntelliCAD software (IntelliCAD Technology Consortium, Portland, OR) and printed at 50,800 dpi at Fineline Imaging (Colorado Springs, CO). A 10:1 weight ratio of PDMS base monomer to curing agent was then mixed, degassed under vacuum, poured onto the patterned wafer to a thickness of 1–2 mm, and cured in an oven at 75 °C for 2 h. After removing the patterned PDMS from the template, through-holes were created at the ends of microchannels by punching the substrate with a manually sharpened syringe needle (NE-301PL-C; Small Parts, Miramar, FL). The patterned PDMS and an unpatterned PDMS piece were cleaned and immersed in a series of solvents for extraction as described below. After extraction, both PDMS substrates were treated with oxygen plasma (PX-250; March Plasma Systems, Concord, CA) for 30 s, assembled together, and placed in an oven at 120 °C overnight to form an irreversible bond and recover hydrophobicity. The microchannel width and depth were both ~20 µm. The integrated nano-ESI emitter was created using an approach similar to that reported previously.²⁵ Briefly, two vertical cuts were made by lining up a razor blade with each of the two guide marks (Figure 1) that were patterned on the device and then pressing the razor blade through the substrates. Five templates that had guide marks arranged at different angles but were otherwise identical were used to study the effect of emitter angle on electrospray stability.

PDMS extraction

Three solvents with different swelling ratios (S) for PDMS were used to extract uncross-linked oligomers and other contaminants from bulk PDMS substrates.³⁰ The patterned PDMS plate with punched holes and a blank PDMS piece were first immersed into 100 mL of highly swelling diisopropylamine ($S = 2.13$) for 2 h with shaking at room temperature. The swollen PDMS was then removed from the diisopropylamine and sequentially placed in 100 mL of toluene ($S = 1.34$) and acetone ($S = 1.06$) for 2 h each. The use of decreasingly swelling solvents minimizes stresses on the substrates and prevents cracking.³⁰ Finally, the PDMS pieces were dried in an oven at 70 °C overnight to completely remove all solvents. The percent of extracted PDMS was determined based on the substrate weights before and after extraction.

Mass spectrometry

MS measurements were performed using an ion funnel-modified³¹ orthogonal time-of-flight MS instrument (Agilent Technologies, Santa Clara, CA). The microchip emitter was positioned 3–5 mm in front of the MS inlet capillary, which was heated to 120 °C. The sample was infused into the PDMS microchip from a 50 µL syringe (Hamilton, Reno, NV) via a fused silica capillary (150 µm i.d., 360 µm o.d.; Polymicro Technologies, Phoenix, AZ) transfer line. The flow rate was controlled by a syringe pump (PHD 2000; Harvard Apparatus, Holliston, MA). To obtain a robust connection between the capillary and microchip, the capillary end was inserted into a ~2-mm-long section of Tygon tubing (TGY-101-5C; Small Parts, Miramar, FL), which was then inserted into the through-hole of the channel on the microchip (Figure 1). The electrospray potential was applied on the syringe needle by a high-voltage power supply. Mass spectra were collected at 5 Hz. The signal-to-noise ratio (S/N) of all mass spectra was calculated

from the equation: $S/N = 2.5S/N_{pp}$, where S was defined as the height of peaks with m/z of interest, and N_{pp} was the peak-to-peak value of the background noise within the m/z range ± 5 amu outside the signal mass range.^{27, 32–33}

Results and discussion

The refinements in fabrication reported here have led to greatly improved performance for integrated PDMS emitters in the nanoflow regime. For example, by incorporating guide marks into the microdevice pattern for accurate cutting (Figure 1), the taper angle at the electrospray emitter could be controlled easily and reproducibly, and we were able to optimize emitter geometry for lower flow rate operation. The effect of the taper angle on electrospray stability has been evaluated previously for similarly shaped microfabricated emitters,^{34–36} but at much greater flow rates than those used here. Figure 2 shows the relative standard deviation (RSD) of total ion signal for a 1 μ M solution of leucine enkephalin as a function of the emitter angle and flow rate. A clear trend toward improved stability with decreasing emitter angle is observed. Electrospray was stabilized at 10 nL/min only for emitter angles $\leq 50^\circ$ and are thus not shown in Figure 2A; the RSDs were 16% and 13% for 30° and 50° tapers, respectively. Representative total ion traces for different flow rates and angles are shown in Figure 2B and 2C. Although the base of the electrospray is not anchored²⁵ to an emitter orifice as is the case with fused-silica capillary emitters, it appears that decreasing the angle at the microchip emitter has effect on electrospray characteristics, including stability and mode of operation,³⁷ similar to decreasing the emitter orifice diameter for conventional emitters.^{38–40} The flow rates achieved here are among the lowest reported for integrated emitters and rival those attainable using commercially available fused silica capillary emitters.⁴¹

The use of highly swelling solvents to extract oligomers and other contaminants from PDMS has previously been used to tailor the surface properties of microdevices (*e.g.*, preserving the hydrophilicity of oxygen plasma-treated PDMS for extended periods).^{30, 42–44} Here, extraction was applied to remove chemical background species and improve the S/N in ESI-MS analyses. Figure 3A shows mass spectra of reserpine using native and extracted PDMS microchips under the same infusion and electrospray conditions. The broad background dominating the mass spectrum in the 300–600 m/z range with the native PDMS chip is magnified in Figure 3B, and most of the background peaks were identified as PDMS oligomers (shown in Table 1). These peaks were essentially eliminated when using the extracted PDMS microchip emitter, and the intensities for the few residual peaks were significantly decreased. The sample peak intensity obtained using the extracted microchip also increased approximately 1.8-fold, which may be attributed to reduced ionization suppression in the absence of contaminants. The S/N for the native PDMS microchip was 233 ± 62 , which increased dramatically to 788 ± 26 following extraction. Figure 3C shows the mass spectra of a mixture containing 6 peptides obtained using an extracted PDMS microchip emitter and a chemically etched capillary emitter,⁴⁵ respectively. The infusion flow rates were the same and the voltages were adjusted to achieve stable electrospray in cone-jet mode. The spectra have similar peak pattern, intensity, and background, indicating that any remaining chemical background peaks likely originated from the electrospray solvent rather than from the PDMS substrates. This was further verified by operating extracted PDMS microchip and capillary-based emitters over a range of different solvent conditions (0 to 50% methanol in water and 10% acetonitrile in water, 0.1% acetic acid in each case) and observing similar performance for both in terms of spray stability and S/N (data not shown).

To optimize the extraction process, the amount of time device substrates were immersed in diisopropylamine was varied from 2 h to 24 h. For 2 h extraction, the substrates decreased in mass by $3.9 \pm 0.2\%$ ($n = 3$) due to the removal of uncross-linked species, and increasing the extraction time to 16 h and 24 h resulted in a respective mass reduction of $5.1 \pm 0.1\%$ and 6.6

$\pm 0.1\%$. The continued decrease in mass with longer exposures indicated that the extraction is incomplete at 2 h. However, there was no clear difference between the mass spectra obtained using PDMS microchips with longer extraction times (data not shown), indicating that despite the incomplete extraction, the remaining contaminants are either too far from the surface or migrate too slowly to interfere with the analysis. In the interest of rapidly prototyping devices, we extracted the PDMS substrates for 2 h in all other experiments.

Another approach reported by others to decrease the background noise for MS applications is to extend the PDMS curing time from the typical $\sim 2\text{--}4$ h to at least 48 h.^{27–29} The extended high temperature treatment can facilitate the cross-linking reactions to reduce the amount of uncross-linked species, but it is not likely to eliminate the unbound species completely. Figure 4 shows the effect of solvent-based extraction relative to the use of extended curing times by comparing mass spectra of angiotensin I using PDMS microchip emitters with different treatments. When the PDMS was thermally treated for 24 h, the MS background noise decreased significantly compared with the native PDMS. However, some noise peaks were still present. By increasing the curing time, the background noise decreased gradually. Although the MS background almost disappeared for the PDMS microchip with curing 72 h, it was still larger than that obtained using extracted PDMS (insets in Figures 4C and 4D). The S/N values of the mass spectra are shown in Figure 4. It is noted that both extraction and thermal treatment approaches can reduce the MS chemical noise generated from the PDMS substrates and increase S/N , but extraction using extremely soluble solvents is more effective than curing for extended times and has the added benefit of consuming less time than the thermal treatment. There may, however, be cases in which solvent extraction is impractical and extended cure times become the most attractive method for reducing contamination from the substrate. For example, solvent extraction has not yet been evaluated for devices containing delicate features such as thin membranes commonly used for valving.⁴⁶

The significant decrease in background noise when using the extracted PDMS microchip emitters directly improved the ESI-MS sensitivity. Reserpine solutions of different concentrations were used as standards to compare S/N for native and extracted microchips. After the PDMS substrates were extracted, the S/N values increased 2–5 times for concentrations ranging from $1\text{ }\mu\text{M}$ to 10 nM (100 nL/min flow rate, not shown). Detection of 1 nM reserpine was only achieved with the extracted devices. We also investigated the performance of the nanospray behavior of native and extracted PDMS microchip emitters over a range of infusion rates. As expected,^{47–48} the signal intensity per unit of consumed analyte increased for both microchips (Figure 5) at lower flow rates, and in agreement with Figure 3A, the reserpine signal intensity was consistently higher for the extracted PDMS microchip. This again provides evidence that removing contaminants not only reduces the observed chemical background, but can enhance the signal by, *e.g.*, reducing charge competition in the ESI process.⁴⁹

Using leucine enkephalin as a model peptide, the achievable sensitivity was determined for the optimized PDMS microchip emitters. As shown in Figure 6A, a 1 nM solution of leucine enkephalin is easily detected when signal averaging, in this case for 1 min, is applied. The periodic noise in the mass spectrum is typical of ESI-MS and has been attributed to solvent clusters.^{50–51} When a single mass spectrum is observed without signal averaging, leucine enkephalin is still clearly detectable (Figure 6B), although the isotopic distribution is less evident. The small number of solution-phase analyte molecules consumed to produce the mass spectrum in Fig. 6B ($\sim 170\text{ zmol}$) points to very low achievable mass detection limits ($\sim 40\text{ zmol}$) as well. Realizing such performance requires not only a highly sensitive mass spectrometer, enabled in this case by the ion-funnel-modified instrument, but also the stable operation at nano-ESI flow rates and minimal chemical background demonstrated in this work.

Conclusion

In this report, we have improved the utility of PDMS microfluidic devices by extraction using a series of solvents to remove the uncrosslinked LMW species and other contaminants in the bulk PDMS. When the extracted PDMS microchip emitters replaced the native ones, the background ESI-MS noise was significantly decreased. A 2h extraction time in diisopropylamine was shown to be sufficient in eliminating most of the chemical background. Compared with a previously used method, curing PDMS devices for several days, the extraction method not only consumed less time, but also provided better results. The decrease in background noise provided significantly higher sensitivity; *e.g.*, a 10 nM detection limit for reserpine was improved to at least 1 nM, and peptide concentrations of 1 nM were also readily detected. As expected, the sensitivity improved at lower flow rates, and by optimizing the emitter geometry, the system could be operated at flows as low as 10 nL/min. It is expected that this optimized PDMS nano-ESI interface will be coupled with on-chip sample handling and separations, which will enable, *e.g.*, proteomics of trace biological samples.

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Figure 1. Schematic illustrations of the PDMS microchip emitter. (Left) patterned PDMS plate including an infusion channel and two guide marks for cutting the emitter arranged at an angle θ . (Right) final PDMS microchip emitter.

Figure 2.

(A) Total ion signal relative standard deviation (RSD) of a 1 μ M solution of leucine enkephalin for different emitter angles and flow rates. (B) Total ion trace of 1 μ M leucine enkephalin at 30 nL/min for a PDMS emitter with an angle of 30°. (C) Total ion trace of 1 μ M leucine enkephalin at 10 nL/min for a PDMS emitter with an angle of 50°.

Figure 3.

(A) Comparison of mass spectra of 1 μM reserpine obtained using native and extracted PDMS microchip emitters. The flow rate was 100 nL/min and the applied voltage was 3300 V. (B) Mass spectrum of the background with m/z ranging from 300 to 600 using native PDMS microchip emitter (shown in the top one of Figure 3A). (C) Comparison of mass spectra of a mixture of 6 peptides obtained using extracted PDMS microchip emitter and capillary emitter. The concentration of each peptide was 1 μM , and the flow rate was 100 nL/min. The voltages applied on the microchip and capillary emitters were 2800 V and 2500 V, respectively. MS peak identifications: m/z 377.78 = syntide 2 $[\text{M}+4\text{H}]^{4+}$; m/z 432.95 = angiotensin I $[\text{M}+3\text{H}]^{3+}$; m/z 503.37 = syntide 2 $[\text{M}+3\text{H}]^{3+}$; m/z 556.33 = leucine enkephalin $[\text{M}+\text{H}]^{+}$; m/z 574.29 = methionine enkephalin $[\text{M}+\text{H}]^{+}$; m/z 587.37 = angiotensinogen 1-14 $[\text{M}+3\text{H}]^{3+}$; m/z 648.91 = angiotensin I $[\text{M}+2\text{H}]^{2+}$; m/z 768.92 = fibrinopeptide A $[\text{M}+2\text{H}]^{2+}$.

Figure 4. Comparison of mass spectra of 1 μ M angiotensin I obtained using three PDMS microchip emitters with different curing times (A–C) and an extracted PDMS microchip emitter (D). The flow rate was 100 nL/min and the applied voltage was 3300 V.

Figure 5. Signal intensity of the 100 nM reserpine peak in the mass spectrum relative to the amount of analyte consumed at different flow rates using extracted and native PDMS microchip emitters.

Figure 6.

(A) Averaged mass spectrum of 1 nM leucine enkephalin in a period of 1 min. The flow rate was 50 nL/min. (B) An individual mass spectrum (0.2 s acquisition) of 1 nM leucine enkephalin at obtained at 50 nL/min using an extracted PDMS microchip emitter.

Table 1

MS peak identifications of the background using native PDMS microchip emitters shown in Figure 3B.

PDMS oligomer structure	<i>m/z</i>	
	[M+H] ⁺	[M+NH] ⁺
$\begin{array}{c} \text{CH}_3 \\ \\ \text{CH}_3-\text{Si}-\text{O}-\left[\begin{array}{c} \text{CH}_3 \\ \\ \text{Si}-\text{O} \\ \\ \text{CH}_3 \end{array} \right]_n-\text{Si}-\text{CH}_3 \\ \\ \text{CH}_3 \end{array}$	n = 3	385.2144
	n = 4	459.2437
$\begin{array}{c} \text{CH}_3 \\ \\ \text{CH}_3-\text{Si}-\text{O}-\left[\begin{array}{c} \text{H} \\ \\ \text{Si}-\text{O} \\ \\ \text{CH}_3 \end{array} \right]_m-\left[\begin{array}{c} \text{CH}_3 \\ \\ \text{Si}-\text{O} \\ \\ \text{CH}_3 \end{array} \right]_n-\text{Si}-\text{CH}_3 \\ \\ \text{CH}_3 \end{array}$	m = 1, n = 3	445.1901
	m = 1, n = 4	519.2197
		462.2197
		536.2487