Capillary-Based Multi Nanoelectrospray Emitters: Improvements in Ion Transmission Efficiency and Implementation with Capillary Reversed-Phase LC-ESI-MS

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We describe the coupling of liquid chromatography (LC) separations with mass spectrometry (MS) using nanoelectrospray ionization (nano-ESI) multiemitters. The array of 19 emitters reduced the flow rate delivered to each emitter, allowing the enhanced sensitivity that is characteristic of nano-ESI to be extended to higher flow rate separations. The signal for tryptic fragments from proteins spiked into a human plasma sample increased 11-fold on average when the multiemitters were employed, due to increased ionization efficiency and improved ion transfer efficiency through a newly designed heated multicapillary MS inlet. Additionally, the LC peak signal-to-noise ratio increased ∼7-fold when the multiemitter configuration was used. The low dead volume of the emitter arrays preserved peak shape and resolution for robust capillary LC separations using total flow rates of 2 μL/min.

The rapid growth in the use of electrospray ionization mass spectrometry (ESI-MS) over the past 2 decades, particularly for biological applications, has been accompanied by ongoing efforts to increase its sensitivity.[1,2] These efforts have been motivated by the fact that at present only a small fraction of the analyte ions ever reach the detector.[3−6] Although ion losses can occur in the mass analyzer, most of the losses presently encountered arise from incomplete droplet desolvation or during transport from atmospheric pressure to the high-vacuum region of the mass spectrometer. Many innovations have been implemented to reduce ion losses at the atmospheric pressure interface, including the use of drying gases,[7] air amplifiers,[8] and electrostatic lenses.[9] In addition, interfaces that replace the standard skimmer with the electrodynamic ion funnel[10−16] have proven far more efficient for transmitting ions through the first vacuum stage.

Besides hardware modifications, operation of ESI at low flow rates can also greatly improve efficiency. The smaller charged droplets produced by an electrospray in the so-called nano-ESI regime facilitate desolvation and increase the amount of excess charge available per analyte molecule.[17−21] Quantitation is thus improved,[20,22,23] and the emitter can be positioned closer to the MS inlet, further reducing ion losses.[1,21] However, problems associated with nano-ESI such as emitter clogging can preclude operation in the nano-ESI regime with its associated benefits for sensitivity. Clogging issues[5,23,24] arise from the internal taper and the requisite narrow orifice of the pulled fused-silica or glass emitters typically employed for nano-ESI. Fortunately, the recent development of chemically etched fused-silica emitters[25] in large

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part solves the clogging issue, as the emitters do not taper internally and relatively large orifices can be used; we routinely operate 20 μm i.d. etched emitters at 20 nL/min in stable cone-jet mode.[26] For liquid chromatography (LC)-ESI-MS analyses, the flow rate delivered to the ESI emitter is generally dictated by the flow from the LC column. As a result, the flow is typically optimized for a particular LC application and not for nano-ESI. Recently developed nano-LC systems aim to increase sensitivity by operating at reduced flow rates. However, very few[27−30] operate optimally below 100 nL/min where the benefits of nano-ESI for sensitivity and quantitation are most pronounced,[22,23] and the nano-LC separations are further limited by a decrease in sample loading capacity and tolerance for dead volume.

Ideally, the flow rates used for LC and for ESI should be chosen and optimized independently, rather than requiring a compromise. Our work seeks to accomplish this by dividing the flow postcolumn among multiple emitters. While multiemitters have been investigated for a variety of proposed applications,[31−36] only recently was their coupling to mass spectrometers demonstrated.[37−39] Since we originally reported a 2−3-fold sensitivity enhancement using a laser-machined polycarbonate microchip having nine discrete emitters,[37] other approaches for improving the sensitivity of ESI-MS analyses with arrays of emitters have been explored. Kim, et al. recently reported microfabricated multiemitters made from Si wafers.[38] This fabrication approach enabled unprecedented line densities of emitters (10 emitters spanning 100 μm) and offered promise for coupling microfluidic separations to MS detectors. The initial demonstration of the Si multiemitters showed only a small improvement for an array of five emitters operated at 600 nL/min relative to a single emitter, which highlighted the need for the MS inlet to be concomitantly modified to efficiently transfer the greater ion current. We recently developed an approach for creating arrays of emitters from chemically etched fused-silica capillaries.[39] The 19-emitter arrays were capable of operation at flows as low as 20 nL/min/emitter. By coupling the emitters with a custom-built MS interface consisting of a multicapillary heated inlet and tandem electrodynamic ion funnels, a 9-fold sensitivity enhancement was observed for reserpine com-

pared to an individual emitter interfaced with the instrument via a single inlet/ion funnel configuration.

Here, we have expanded the characterization of the capillary-based emitter arrays and have further improved ion transmission efficiency by ~60% with a redesigned multicapillary heated inlet. In addition, arrays of emitters have been used for LC-ESI-MS analyses for the first time. The LC-ESI-MS experiments demonstrated sensitivity gains greater than an order of magnitude for peptides from proteins spiked in human plasma and showed substantial gains in signal-to-noise ratio (S/N) for extracted LC peaks. For the capillary LC separations, which were operated at ~2 μL/min, peak width and shape were preserved when the emitter arrays were employed due to their low internal volume and their ready coupling to the LC columns.

EXPERIMENTAL SECTION

Sample Preparation. Acetonitrile (ACN) and 49% hydrofluoric acid were purchased from Fisher Scientific (Fair Lawn, NJ). Glacial acetic acid (HAc) and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich (St. Louis, MO). Water was purified using a Barnstead Nanopure Infinity system (Dubuque, IA). Mobile phases A and B, which were used for the gradient elution LC separations, consisted of H_2O/HAc/TFA (100:0.2:0.05; v/v/v) and ACN/H_2O/TFA (90:10:0.1; v/v/v), respectively. A standard peptide solution was used for direct infusion experiments that contained angiotensin I, neurotensin, bradykinin, and kemptide (Sigma-Aldrich). The peptide concentrations were 500 nM each, and the solvent was 2:1 mobile phase A/mobile phase B.

Preparation of the spiked human blood plasma sample used for the LC experiments has been described in detail elsewhere.[40] Briefly, human blood plasma obtained from a healthy volunteer was determined to have an initial protein concentration of 65 mg/mL. Bovine carbonic anhydrase II, rabbit glyceraldehyde-3-phosphate dehydrogenase (G3PDH), *E. coli* β-galactosidase, and bovine β-lactoglobulin (Sigma) were spiked to a concentration of 40 μg/mL each into the unprocessed human plasma. Equine skeletal muscle myoglobin and chicken ovalbumin (Sigma) were spiked at 4 μg/mL. The spiked sample was depleted of 12 high-abundance plasma proteins using an IgY-12 affinity LC column (Beckman Coulter, Fullerton, CA) and then tryptically digested, fractionated by strong cation-exchange chromatography, dried under vacuum, and dissolved in 30 μL of 25 mM NH$_4$HCO$_3$ as is typically done for such analyses at our laboratory. For this work, 14 of the fractions were pooled, and the sample was further diluted in mobile phase A to a final peptide concentration of 0.1 μg/μL.

ESI Emitters. Individual emitters were made from ~4 cm sections of 20 μm i.d. × 150 μm o.d. fused-silica capillaries (Polymicro Technologies, Phoenix, AZ) and were chemically etched using a previously published procedure.[25] Multiemitter arrays were made from 19 fused-silica capillaries (20 μm i.d. × 150 μm o.d.) as described previously,[39] but with the following modification: Devcon HP250 epoxy (Danvers, MA) was used to seal around the individual capillaries in place of Devcon 14250

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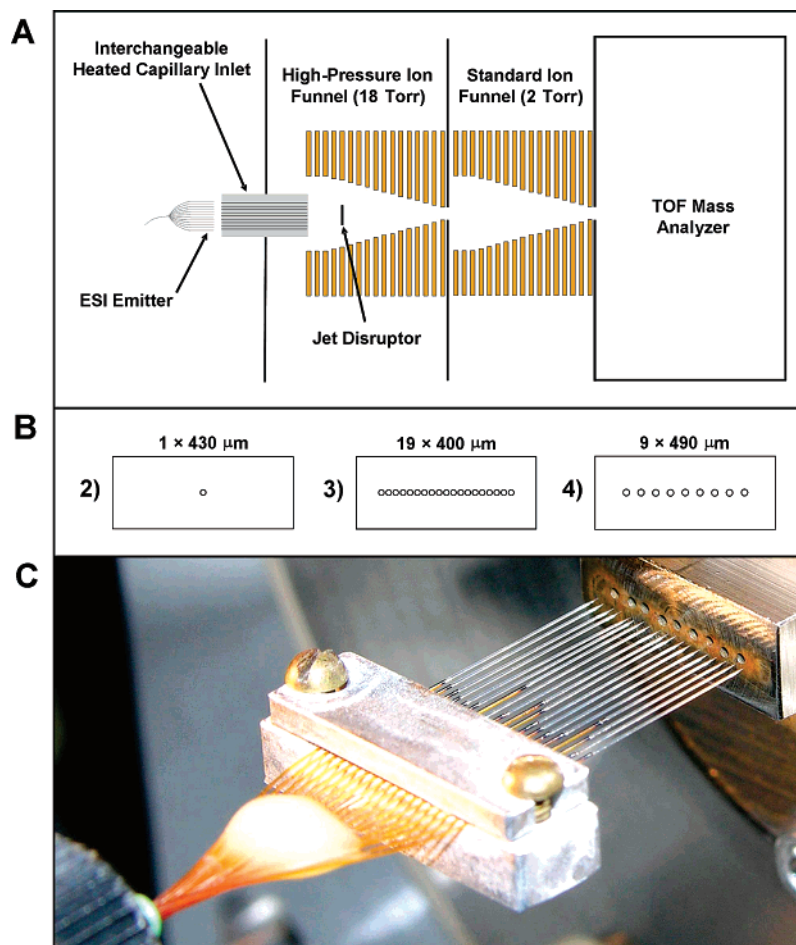
Figure 1. Experimental setup. (A) Schematic depiction of the TOF instrument modified with an interchangeable heated capillary inlet and differentially pumped tandem ion funnels. The entrance and exit diameters of both the high-pressure and standard ion funnels were 25.4 mm and 2.5 mm, respectively. (B) Front view of the heated capillary inlets used for instrument configurations 2−4 (Table 1). Capillary spacing (center-to-center) was 500 μm in configuration 3 and 1.0 mm in configuration 4. (C) Photograph of an array of 19 emitters positioned in front of a heated multicapillary inlet (configuration 4).

Table 1. Summary of Emitter/Inlet Combinations That Were Evaluated

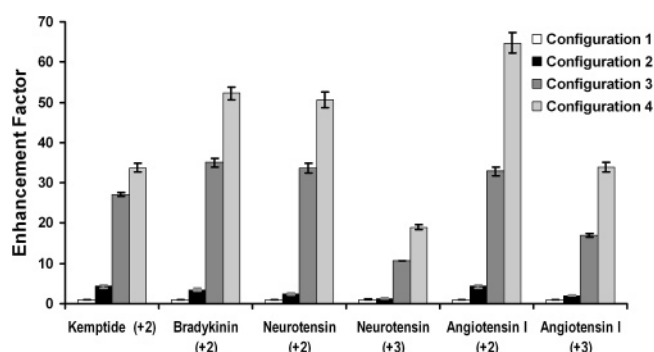| configuration	emitter	MS interface
1	1 × 20 μm i.d.	standard Agilent NanoESI
2	1 × 20 μm i.d.	heated capillary (1 × 430 μm i.d.) /tandem ion funnel
3	19 × 20 μm i.d.	heated capillary (19 × 400 μm i.d.) /tandem ion funnel
4	19 × 20 μm i.d.	heated capillary (9 × 490 μm i.d.) /tandem ion funnel

Figure 2. Sensitivity comparison of a mixture of standard peptides for the four different emitter/inlet configurations in Table 1. The solution flow rate was 2.0 μL/min. Additional description is in the text.

epoxy. The epoxy was mixed according to the manufacturer's specifications and cured at 80 °C for 2 h after being applied to the devices.

MS Instrumentation. All MS experiments used an Agilent 6210 (Santa Clara, CA) time-of-flight (TOF) mass spectrometer, either with Agilent's commercially available NanoESI interface or a tandem ion funnel interface, which is depicted in Figure 1A. The tandem ion funnel interface accommodated the increased gas throughput from the multicapillary inlets and used the same design described previously.[16,39] The high-pressure ion funnel, which was operated at 18 Torr for this work, was driven at 1.7 MHz with an amplitude of 170 V_{p-p}. The conventional ion funnel operated at 1.3 Torr with an rf of 730 kHz at 100 V_{p-p}. A linear dc

gradient of 17.0 V/cm was applied to the 18 Torr ion funnel, and 14.8 V/cm was applied to the 1.3 Torr ion funnel.

Four ESI source configurations were evaluated as outlined in Table 1. In addition to the standard Agilent NanoESI interface used with an individual ESI emitter (configuration 1, Table 1), three different heated capillary inlets, depicted in Figure 1B, were used at different times with the ion funnel-modified TOF mass spectrometer. The single-capillary inlet (configuration 2) was 6.4 cm long and had an i.d. of 430 μm. The 19-capillary inlet
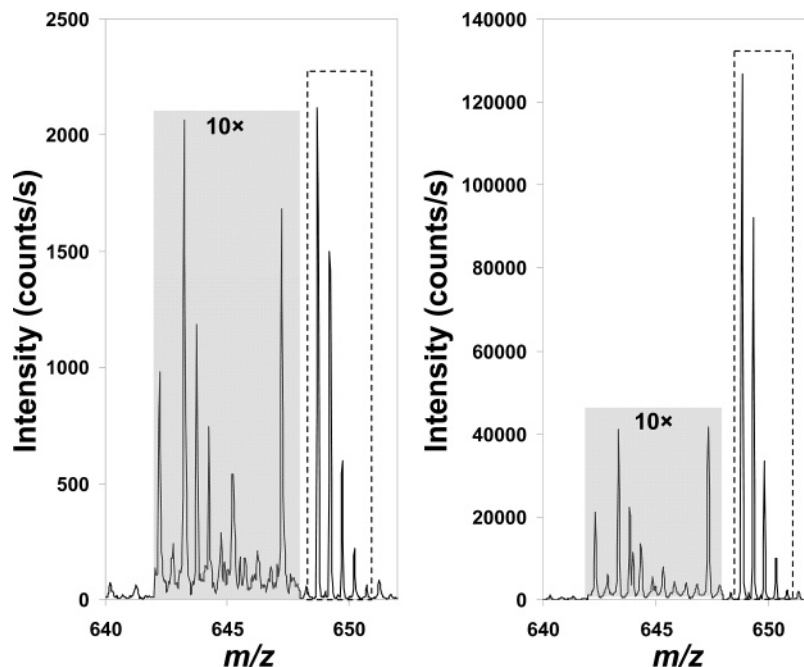
Figure 3. Mass spectra of angiotensin I $[M + 2H]^{2+}$ (inside of dashed lines) from (left panel) configuration 1 and (right panel) configuration 4. The shaded baseline to the left of the analyte signal was magnified 10-fold for clarity.

(configuration 3) had the same length and was comprised of 400 μm i.d./500 μm o.d. capillaries (part no. 88935K236, McMaster-Carr, Los Angeles, CA) with 500 μm center-to-center spacing. The final inlet evaluated consisted of a linear array of nine inlet capillaries (configuration 4) that was fabricated in a stainless steel body using electrical discharge machining. The 490 μm i.d. inlets were spaced 1.0 mm on center and were 4.4 cm long. Figure 1C shows the multi-ESI array interfaced with the nine-capillary inlet. The inlets from configurations 2–4 were heated to 125 °C and were biased 10 V higher than the first ion funnel electrode. For all experiments other than those employing the Agilent NanoESI source, the individual or multiemitters were positioned 1–1.5 mm from the inlet, and a 2 kV potential was applied to the solution through the stainless steel union that connected the LC column or infusion line to the emitter. When the Agilent NanoESI source was used, the emitter position was optimized for maximum signal intensity and an ESI potential of 1.8 kV was used. A counterflow of heated N_2 was applied at 250 °C and 7.0 L/min.

Infusion data were acquired in profile mode, and signals were averaged for 30 s (10 000 cycles/scan, 0.89 scan/s). For LC separations, data were collected in centroid mode with the minimum thresholding allowed by the software (50 counts).

Liquid Chromatography. Reversed-phase capillary LC separations with an exponential solvent gradient[41] were performed using a Gilson 321 pump (Middleton, WI). The adjustable solvent mixer on the pump was set to 2.2 mL, and an external six-port valve (VICI Valco, Houston, TX) with a 10 μL sample loop was used for sample loading and injection. A 20 μm i.d. × 360 μm o.d. open tubular fused-silica capillary (Polymicro Technologies) served as a microflow splitter that was adjusted in length such that the flow rate through the LC column was 2 μL/min when

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the pump flow rate was set to 21 μL/min. Operational pressure was ~5000 psi. The 65 cm long, 150 μm i.d. fused-silica analytical columns were packed in-house with 5 μm porous C-18 bonded particles (Phenomenex, Torrance, CA) as described previously.[41]

RESULTS AND DISCUSSION

In our previous work, direct infusion results were compared between ESI source configurations 2 and 3 (Table 1). Here, we have expanded the comparison to include two additional configurations. Configuration 1 enabled the instrument modifications of the present work to be compared with the unmodified TOF mass spectrometer. Changing between configurations 1 and 2 replaced the skimmer with the ion funnel and positioned the ES emitter axially instead of orthogonally to the MS inlet. Also, configuration 1 used a heated curtain gas to assist in desolvation, whereas a heated capillary inlet was employed for configurations 2–4. By contrast, when comparing between configurations 2–4, only the emitters and the inlets were changed, and all other factors, including pressures in the vacuum chambers, the distance from the emitters to the inlets, the ESI voltage, etc., were held constant. It was determined experimentally that an applied ESI voltage of 2.0 kV with 1–1.5 mm spacing from emitter to inlet optimized measured ion currents for configurations 2–4. Figure 2 presents relative peak intensities for direct infusion of a mixture of four peptides using the four different configurations. Peak intensities were normalized such that those for configuration 1 were unity. Thus, the plot shows "enhancement factors" relative to configuration 1. Error bars in Figure 2 are standard deviations based on three replicate analyses for which the signal was averaged for 30 s each time. Of particular note in Figure 2, configuration 4, which was developed for this work in an effort to further improve ion transmission efficiency, provided an ~60% improvement relative to configuration 3. This was due to the larger inlets and the decrease in total length, which reduced ion losses to the interior
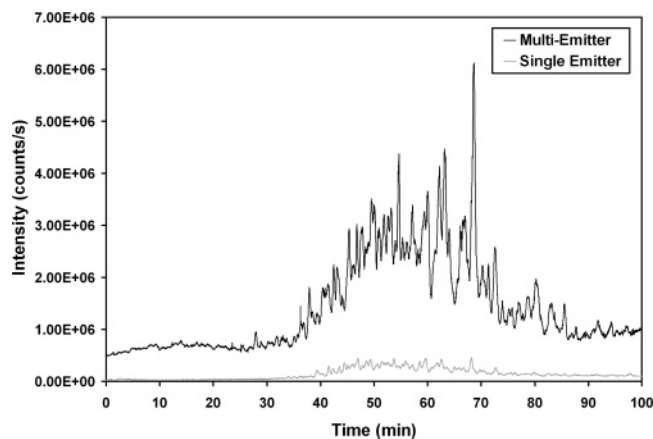
Figure 4. Total ion chromatograms of depleted human plasma (1 μg total sample) using a multiemitter (configuration 4) and a single emitter (configuration 2).

Figure 5. Signal enhancement for 10 peptides spiked into depleted human plasma tryptic digest. Peptide I.D.s are listed in Table 2. Additional description is in the text.

walls of the inlet capillaries.[6] Remarkably, the combined enhancements of the ion funnel, the emitter arrays, and the improved ion transmission efficiency produced an increase in analyte peak intensities averaging more than 40-fold relative to the unmodified TOF MS instrument.

Although the sensitivity gains demonstrated in Figure 2 are impressive, improvements in S/N were more modest. For the four peptides analyzed here, we observed an average improvement in S/N of just 80% between configurations 1 and 4. The reason for the small gain was that S/N for this experimental setup was limited by chemical, rather than random, noise. This is demonstrated in Figure 3, which shows the mass spectrum for angiotensin I with the adjacent baseline that was used to calculate S/N magnified 10-fold. The "noise" peaks in the spectra match, indicating that they are not random. Further, S/N did not change for configuration 4 even when signal averaging was decreased from 30 to 1 s (not shown). For cases in which S/N is dominated by chemical noise, the benefits of the multi-ESI approach are limited, as detection of contaminating species is enhanced along with the analytes of interest. However, the increased ion currents should still benefit MS/MS experiments because the decrease in ion trap fill times will reduce undersampling. Additionally, as chemical noise is generally singly charged[42] and most electrosprayed peptides are multiply charged, a technique that is capable of spatially separating ions according to charge state (e.g., ion mobility spectrometry (IMS)-TOF MS[43]) should allow the increased ion signal afforded by this technique to translate to improved S/N and reduced detection limits.

In addition to being further characterized for direct infusion applications, the emitter arrays were also evaluated in conjunction with LC for the first time. Figure 4 shows chromatograms of tryptically digested depleted human plasma when a single emitter (configuration 2) and a multiemitter (configuration 4) were used. Comparison between these two configurations enabled all instrument parameters to be held constant apart from the emitters and the inlets. The chromatograms are plotted on the same intensity scale to show the marked increase in sensitivity afforded by the
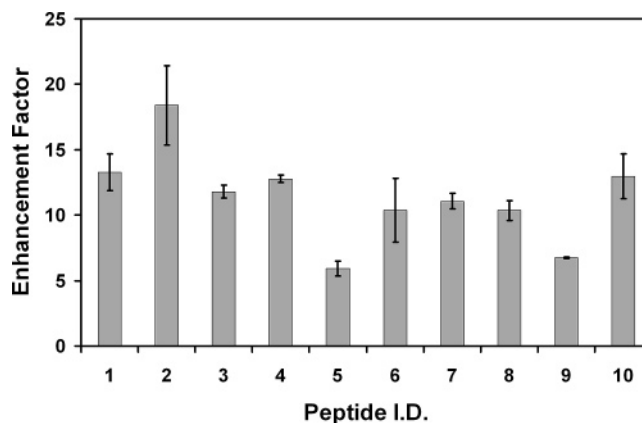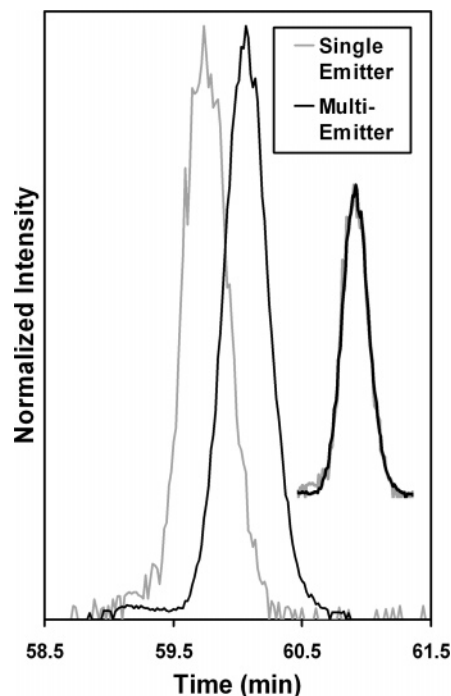

Figure 6. Extracted ion chromatograms of peptide 10 (Table 2) from analyses employing a single emitter and an array of 19 emitters. The peaks are overlaid in the inset, showing identical peak shapes and widths.

emitter array. Intensities of individual peptides from spiked proteins were also identified and compared between the two configurations, and the signal gains from configuration 2 to configuration 4 are presented as enhancement factors in Figure 5; error bars are standard deviations based on three replicate separations. Figure 5 shows that signal intensities increased on average more than 11-fold, somewhat higher than our previous report,[39] due to the improved ion transmission efficiency through the modified inlet.

It is vital that the improvements in ion production and transmission afforded by multiemitters do not degrade chromatographic resolution due to postcolumn peak broadening. A benefit of our capillary-based emitters is that they connect to capillary columns using standard fittings. Coupling other reported emitter arrays[37,38] to LC would not be so straightforward. Nevertheless, a

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Table 2. Peptides from Proteins Spiked in Human Plasma That Were Used for Sensitivity Comparisons

peptide I.D.	m/z	sequence	protein
1	1127.08	YGDFGTAAQQPDGLAVVGVFLK	bovine carbonic anhydrase II
2	791.41	YAAELHLVHWNTK	bovine carbonic anhydrase II
3	882.4	LISWYDNEFGYSNR	rabbit G3PDH
4	908.45	GLSDGEWQQVLNVWGK	horse myoglobin
5	636.34	LFTGHPETLEK	horse myoglobin
6	887.45	ISQAVHAAHAEINEAGR	chicken ovalbumin
7	697.87	LPSEFDLSAFLR	*E. coli* β-galactosidase
8	871.95	LSGQTIEVTSEYLFR	*E. coli* β-galactosidase
9	684.33	YHYQLVWCQK	*E. coli* β-galactosidase
10	600.83	LDQWLCEK	bovine β-lactalbumin
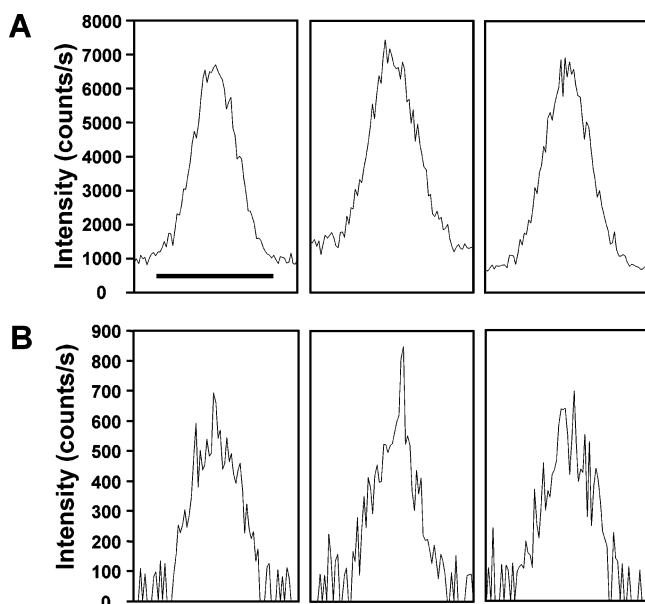
Figure 7. Extracted ion chromatograms of peptide 7 (Table 2) from three replicate separations using (A) configuration 4 and (B) configuration 2. Scale bar in the left panel of (A) equals 1 min and applies to all panels.

potential concern still existed, as the i.d. of each capillary in the array was the same as the i.d. of the single emitter used for comparison. Because the emitter lengths were similar, the internal volume of the emitter array was ~20 times greater than that of the single emitter. We evaluated extracted ion chromatograms (EICs) from the two configurations to compare peak widths and shapes. Figure 6 shows the EICs for peptide 10 (Table 2) when the single and multiemitters were used. By normalizing the peaks for height, it is clear that they are essentially identical in terms of peak width and shape, and therefore postcolumn broadening did not significantly impact this separation. Postcolumn broadening could become a greater contributor to peak width for lower flow rate separations, in which case smaller i.d. emitters may be attractive. However, the present results show that good chromatographic fidelity can be obtained at flow rates close to the optimum for high separation efficiency.

Although the peaks in Figure 6 match well in terms of overall shape and width, the peak from the separation employing the multiemitter demonstrates a greater S/N. The effect of the emitter arrays on chromatographic S/N was further explored by examining a less abundant spiked peptide. The replicate EICs of peptide 7 (Table 2), which gave a ~10-fold lower signal than the peptide

in Figure 6, are shown in Figure 7. A 0.5 Da mass window centered on the monoisotopic mass peak was used for obtaining the EICs to account for small shifts in observed m/z. Analysis of the S/N from three replicates indicate a nearly 7-fold enhancement. Both the greater signal intensities and the improved ESI stability[39] of the multiemitters likely contribute to the dramatically improved S/N.

CONCLUSIONS

For the first time, we have demonstrated the efficient coupling of emitter arrays with capillary LC separations for enhanced sensitivity. The separations showed no loss in peak shape or resolution relative to separations employing a single ESI emitter, due to the low dead volume of the emitters and their facile coupling to the LC column. A sensitivity enhancement averaging 11-fold was observed for peptides from spiked proteins in a tryptic digest of depleted human plasma. The dramatic sensitivity gains were made possible by the increased ionization efficiency of the multiemitters, along with efficient transfer of ions into the mass spectrometer. A new heated multicapillary inlet developed for this work proved ~60% more efficient for ion transfer relative to our previous design.[39] Chromatographic S/N improved nearly 7-fold with the use of multiemitters, whereas the S/N gains for individual mass spectra were more modest due to chemical noise limitations. However, coupling the increased signal afforded by this approach with efficient spatial separation of singly charged species (using, e.g., IMS-TOF MS) should dramatically improve detection limits for multiply charged peptides, even when an analysis would otherwise be chemical noise-limited. Additionally, we expect that the multiemitters will enable higher dynamic range LC−ESI-MS analyses by combining the greater sample loading capacity[41] of larger capillary columns with the sensitivity that is otherwise only achievable with smaller i.d. columns operating in the nanoflow regime.