APPLICATION NOTE

A Simple Nanoelectrospray Arrangement With Controllable Flowrate for Mass Analysis of Submicroliter Protein Samples

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A simple arrangement for nanoelectrospray ionization using a conventional syringe pump connected to a pulled unmodified capillary has been evaluated. This arrangement avoids several disadvantages associated with metal-coated nanoelectrospray emitters. The relatively large orifice (~9 μm) at the pulled capillary tip reduces sample clogging and the use of the pump minimizes spray disruption due to gas bubbles. Subattomole detection limit was achieved with nanomolar protein sample solutions at 5–10 nL/min flowrates using an LCQ mass spectrometer. Submicroliter samples can be loaded from the tip orifice and stored inside the capillary to virtually eliminate any dead volume, and then be electrosprayed for extended periods at well-controlled flowrates. (J Am Soc Mass Spectrom 2000, 11, 94–99) © 2000 American Society for Mass Spectrometry

Electrospray ionization mass spectrometry (ESI-MS) was first demonstrated by Yamashita and Fenn [1], as well as by Aleksandrov and co-workers [2] in 1984. Since the late 1980s, ESI-MS has developed into a powerful analytical technique for the analysis of macromolecules and biological materials [3–5]. In early efforts, the inner diameter (i.d.) of electrospray emitter orifice was generally 50 to 250 μm, with sample solution direct infusion flowrates of several μL/min or higher [6, 7]. The first nanoelectrospray (solution flowrate at nL/min range) analyses were actually conducted in early CE/MS interfacing efforts [8, 9], the deliberate ESI-MS for direct infusion of protein and oligonucleotide samples at submicroliter per minute flowrate (0.05–0.5 μL/min) was first described in 1993 by Gale and Smith [10]. Subsequently, Caprioli and co-workers achieved sub-nM concentration detection limit for neurotensin at 0.82 μL/min flowrate using their microelectrospray source [11]. Wilm and Mann later reported and popularized a nanoelectrospray strategy using gold-coated pulled capillary emitters [12–15]. Using similar tips, Valaskovic et al. achieved attomole sensitivity for protein characterization at very low flowrates, ~1–4 nL/min [16, 17].

These studies have resulted in widespread use of nano-ESI-MS, and the key to success being the low infusion flowrate at which the sample is delivered to the electrospray tip orifice. The majority of previous and recent studies have been conducted using fused silica or glass capillary emitters having gold or silver coatings [18], with the electrospray voltage applied via the conductive coating. For off-line analysis, samples were almost always loaded from the distal end of the emitter, sometimes using pressure to shorten loading times [13, 19]. However, metal-coated capillary emitters have some inherent disadvantages. First, to maintain a stable nanoelectrospray a small tip orifice, ~1–2 μm i.d., is often needed. Particle clogging is thus problematic. Second, the metal coating at the capillary tip often degrades during ESI [20–23]. "Tip touching" against a surface to re-open a blocked orifice, which can sometimes re-initiate nanoelectrosprays [13, 24–26], can also result in damage to the metal coating, or break the tip. Third, gas bubbles formed at, or transported to the orifice, can often abruptly terminate a nanoelectrospray (due to the interruption of tip capillary action), and can be difficult to remove without the use of pressure. Fourth, the retrieval of unused sample is often impractical. Fifth, the actual nanoelectrospray flowrate is typically determined after a certain volume of sample is analyzed over a period of time, and the reproducibility of this flowrate is generally poor (with as much as 50% uncertainty) once the emitter or the sample is changed. Finally, the cost of such emitters (typically not reused), either purchased from commercial sources or manufactured in house, is much higher than conventional unmodified pulled capillaries.

There are various alternatives for generating nanoelectrosprays without using metal-coated emitters [27–33]. Here we describe implementing a simple design

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Table 1. LCQ acquisition settings for various nanoelectrospray studies

Type of study	Ion injection time (ms)	Number of microscans	Automatic gain control (AGC)	Scan data type
Pump on and off	10–100	3	On	Profile
Detection limits	10–100	1	Off	Profile
Microliter analysis	20–50	1	On	Centroid

using uncoated pulled capillaries with a larger tip orifice that overcomes the disadvantages of the metal-coated capillary emitters while maintaining comparable detection limit for nanoelectrospray mass spectrometry. By the simple expedient of directly connecting a conventional syringe with a pulled capillary, the flowrate for sample delivery can be accurately controlled at much lower flowrates (5–50 nL/min) than conventionally operated. The transparent tip region also allows direct viewing so that proper action(s) with irreplaceable samples can be taken upon signal interruption (i.e., sample is not necessarily lost). Because of the pressure-driven flow, gas bubbles are effectively expelled from the tip during analysis. This arrangement also facilitates loading submicroliter sample volumes from the capillary tip, rather than from the distal end, and stores the sample only inside the capillary, so that no sample losses occur at any junction, contamination is minimized and unused sample can be retrieved. Standard protein samples were used to characterize the nanoelectrospray properties of the arrangement and to demonstrate its application for analysis of submicroliter samples.

Experimental

Commercially available proteins and solvents, cytochrome *c* (horse heart), myoglobin (horse heart), lysozyme (chicken egg white), carbonic anhydrase (bovine erythrocytes), methanol (HPLC grade), and glacial acetic acid (ACS reagent) were purchased (Sigma Chemical, St. Louis, MO) and used without further purification or microfiltration. Protein solutions were prepared using freshly generated 18.3 MΩ nanopure water (Barnstead, Dubuque, IA) and diluted into desired concentration with final solution composed of H_2O/MeOH/HAc (v/v 50/49/1). Pure solvent liquid of the same composition (without protein) was also prepared for background reference and used in submicroliter sample analysis as a conductive "filling" solvent (see below). Fused-silica capillary tubing (Polymicro Technologies, Phoenix, AZ) was pulled with a CO_2 laser micropipet puller (Model P-2000, Sutter Instruments, Novato, CA). Data presented were obtained with capillaries (365 μm o.d. × 100 μm i.d.) pulled to 9 ± 1 μm i.d. at the tip orifice with the puller program settings at 350, 0, 30, 120, and 0, for heat, filament, velocity, delay, and pull parameters, respectively. The program was looped twice and the dimensions of the pulled capillary tip orifice were examined under an optical microscope with up to 1000 × magnification (Model BX60, Olympus, Japan). The butted distal end of the pulled capillary (cut to 5–25 cm long) was connected to a 10 or 25 μL syringe (Hamilton, Reno, NV) through a finger-tight peek union and appropriate peek sleeves (Upchurch Scientific, Oak Harbor, WA). The syringe pump (Model 22, Harvard Apparatus, Holliston, MA) was operated at 5, 10, 20, 50, and 200 nL/min (flow accuracy experimentally confirmed). The electrospray high voltage (1.1–1.4 kV) was applied at the metal syringe needle and the capillary tip was placed 0.5–2 mm in front of the heated capillary inlet (at 180 °C) of the mass spectrometer, an LCQ ion trap mass spectrometry (Finnigan, San Jose, CA), with an X–Y–Z micropositioner (Coherent, Auburn, CA). For nanoelectrospray property studies, the sample was loaded into the syringe before it was connected to the pulled capillary. For microliter and submicroliter sample analyses, the "filling" solvent was first loaded into the syringe, which was then connected to a 25-cm long pulled capillary. The conductive solvent was allowed to fill the entire system (capillary, peek union, syringe barrel) before aspirating the protein sample solution through the capillary tip orifice. Mass spectra were obtained using the LCQ instrument in mass spectrometry full scan mode with different acquisition settings as shown in Table 1.

Results and Discussions

Nanoelectrospray Behaviors at Spray Initiation and Termination

Earlier studies using syringe pumps have reported the use of nanoelectrosprays at 10 [21], 33 [33], and 50 nL/min [10] by direct infusion with 20 to 25 μm i.d. tips. At low flowrates, the stepping motor of the pump pulses the syringe forward at intervals on the time scale of a few seconds. However, because of the resistance provided by small i.d. tubing, we found that the simple nanoelectrospray arrangement could provide stable signals extending to over 1 h. To better understand the nanoelectrospray stability issues and its characteristics, we examined spray initiation and termination as the pump was turned on and off at various flowrates. Taking relatively concentrated cytochrome *c* solution (15 μM) as an example, there was initially no significant total ion current (TIC) at time zero when the syringe pump was just turned "on" at 5 nL/min flowrate (time 0, Figure 1A). The nanoelectrospray was initiated after 1.8–1.9 min and mass spectral signal quickly stabilized

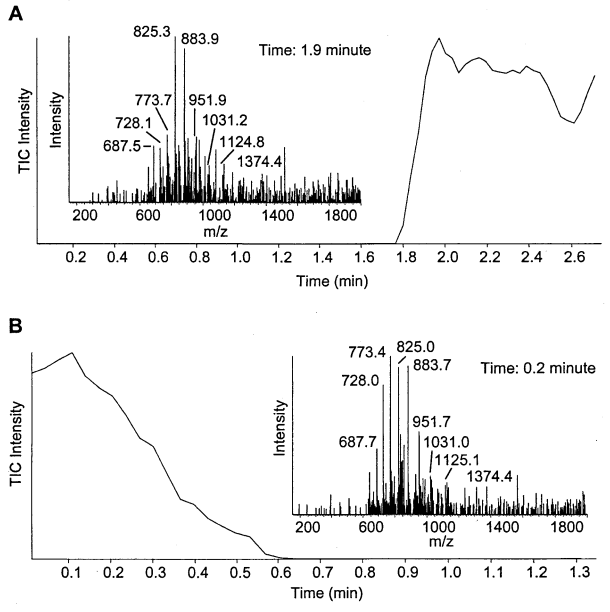

Figure 1. Total ion current (TIC) of nanoelectrospray at 5 nL/min flowrate for direct infusion of a 15 μM cytochrome *c* solution (data acquired at 2 s/scan rate). (**A**) Syringe pump turned "on" at time = 0 min and (**B**) syringe pump turned "off" at time = 0 min. Insets show mass spectra at various times.

(with minor fluctuation) as shown in the insert where charge states due to the protein are clearly evident. Similar single scan spectra were recorded over an extended period, indicating that nanoelectrosprays at this flowrate were stable. The delay time was largely dependent on the flowrate and it varied slightly even under the same conditions. As expected, a higher flowrate results in a shorter delay time (e.g., at 200 nL/min, time $<$ ~10 s).

Earlier studies have shown the influence of tip geometry (e.g., i.d., tapered, or butted end) on nanoelectrospray behaviors [16, 21, 33–37]. The i.d. of the tip orifice and whether it is tapered (as a result of capillary pulling) affect initiation and spray stability. We obtained stable nanoelectrospray for flowrates as low as 10 nL/min using capillaries with tips pulled to around 20 μm i.d., similar to a previous study [21]. Typically, pulled capillaries with a ~10-μm i.d. tip will initiate and maintain a stable spray at $>$ 5 nL/min flowrates. However, nanoelectrospray can become unstable if too high an electrospray voltage is applied [36].

At low flowrates, even when the pump is "on," the pressure results from the brief intervals (a few seconds) during which the syringe is advanced. It is useful to evaluate electrospray while the pump is completely turned off, the result of which can help to explain the stability of the nanoelectrospray between pump pulses. Spray behavior after the pump is turned "off" (i.e., flow stopped, time 0, high voltage still on) was also monitored (Figure 1B) and it was found that signal persisted for another 30 s. The mass spectrum (inset, Figure 1B) at 0.2 min after the pump was turned off showed virtually no difference from earlier spectra (pump on, time $<$ 0). The TIC was often observed to decrease quickly in the first tens of seconds, during which it was composed of mostly ionized protein molecules. Chemical noise and contributions due to some low molecular weight impurities would often persist several minutes after the pump was turned off (especially at initially applied higher flowrates), even though the relative intensity of this residual "signal" was often two orders of magnitude smaller.

Using a microscope, it was observed that, in this tightly sealed system, liquid movement inside a capillary stopped almost instantaneously when the pump was turned off at 5–200 nL/min flowrates. However, tip capillary action, the electric field [12], and the voltage drop across the capillary (i.e., between the syringe needle and the tip orifice), can result in continued electrophoretic delivery of analyte ions, and assist in maintaining the nanoelectrospray. These observations imply that there is actually a highly nonuniform flowrate being provided to the tip during operation with the pump on. The effects due to this need to be further examined because it is possible that the spectrum quality for these situations may vary significantly. Other factors (e.g., ion production rate, analyte ionization efficiency, sample concentration, solvent evaporation rate) and details that determine the shape and stability of the Taylor cone are also likely to affect the initiation, the intensity and stability of the nanoelectrospray, and the duration of the lingering spray after flow is stopped. Studies for flowrates of 5, 10, 20, 50, and 200 nL/min using different protein samples at various concentrations gave similar trends (data not shown), and demonstrated that it is practical to achieve nanoelectrospray with this simple arrangement.

Nanoelectrospray Detection Limits

A major advantage of nanoelectrospray is its low detection limits, in terms of both molar concentration of the analyte in solution and the absolute amount of analyte consumed. Clearly, the latter also depends on the type of mass analyzer and mode of scan used. For ion trapping instruments, such as the LCQ, tuning parameters (e.g., ion injection time) can be adjusted so that only a small portion of the electrosprayed ions are allowed to enter the mass spectrometer. Therefore, the mass spectra obtained represent the detection of a fraction of the continuously ionized molecules that are consumed in each ion injection, manipulation, and detection cycle. In the discussion regarding a particular averaged spectrum, the actual sample consumption over the entire time for spectrum generation, and the analytical detection limit for the sample are different as indicated in the following eqs 1 and 2:

$$\text{Sample consumption} = N \times C \times R \times Ts \quad (1)$$

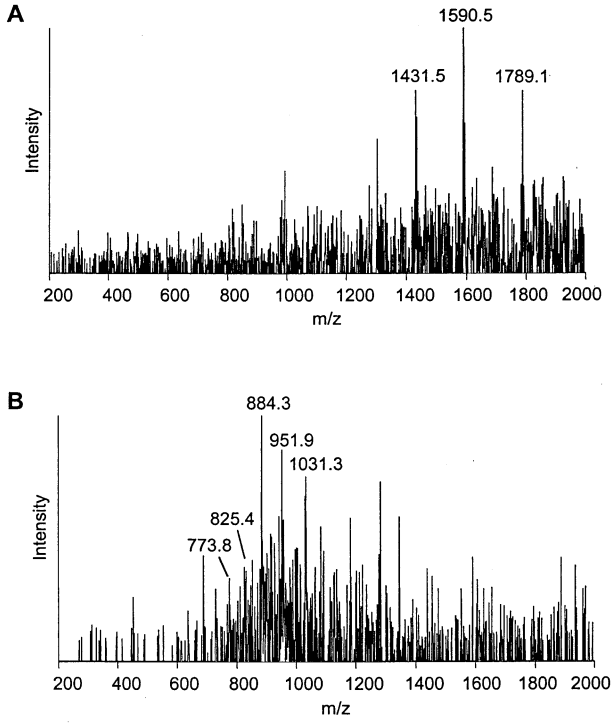

Figure 2. Mass spectra of subattomole detection limit (average of 3 scans) for (**A**) 35 nM lysozyme (measured molecular weight, meas. MW. 14,305 ± 1 Da) at 10 nL/min flowrate, 16 amol consumed (0.9 s/scan), 0.9 amol injected into the ion trap (ion injection time: 50 ms/scan); and (**B**) 150 nM cytochrome *c* (meas. MW. 12,359 ± 1 Da) at 5 nL/min flowrate, 34 amol consumed (0.9 s/scan), 0.4 amol injected into the ion trap (ion injection time: 10 ms/scan).

$$\text{Detection limit} = N \times C \times R \times Ti \quad (2)$$

in which N is the number of scans averaged; C, sample molar (or mass) concentration; R, flowrate; Ts, overall time needed per scan; and Ti, ion injection time in each scan. Although Ts varies relatively little (e.g., ~1 s/scan), the ion injection time, Ti, varies greatly.

Figure 2A shows that subattomole detection limit, 0.9 amol (calculated from eq 2, $N = 3$, $Ti = 0.05$ s), for 35 nM lysozyme solution can be achieved at 10 nL/min flowrate, with an ion injection time of 50 ms (comparing to $Ts = 0.9$ s). Similarly, for the 150 nM cytochrome *c* solution, the obtained averaged spectrum represents an analytical detection limit of 0.4 amol protein (Figure 2B). The actual sample consumption was 16 and 34 amol, respectively (eq 1); apparently, less than a few percent of generated ions were used for the mass spectral data averaging. In the LCQ, Ti is generally a small fraction of Ts (in Figure 2, $Ts = 0.9$ s for both cases), but additional sensitivity gains cannot be obtained simply by increasing Ti due to space-charge limitations and ion trapping capacities of the instrument. We were able to detect ~20 nM lysozyme and cytochrome *c* samples (data not shown) under optimal conditions. Low-attomole and subattomole detection limits for proteins have been demonstrated using Fourier transform ion cyclotron resonance mass spectrometry [16, 17], and we have recently demonstrated detection limits estimated at 30 zmol using 100 nL/min flowrate [38]. Low nM concentration detection limit was previously reported for smaller peptides using an LCQ [39]. Our results demonstrate subattomole and 20 nM concentration detection limits for protein samples using an LCQ mass spectrometer, illustrating the high potential for trace level detection.

Submicroliter Sample Analysis

An attraction of this nanoelectrospray arrangement is the direct introduction of sample from the tip of the pulled capillary. The larger i.d. of the tip orifice reduces the chances of particle clogging when sample solution is flowing out of the capillary (syringe pushing), or when it is drawn into the capillary from the outside (syringe pulling). The conductive solvent used to fill the capillary and syringe ensures uninterrupted electric continuity as the sample solution is drawn into the tip, continuously displacing the solvent. A 25 cm long, 100 μm i.d. pulled capillary (with a few mm long taper) has an internal volume of ~2 μL. Diffusion at the interface between sample solution and the solvent inside the capillary is expected to be small for the typical analysis time (<1 h), consistent with experimental observations (Figure 3A).

Figure 3A (TIC) and 3B (averaged mass spectrum) show the results for the analysis of 1 μL of 130 nM carbonic anhydrase solution. At 50 nL/min flowrate, spectra were obtained for 20 min (over 1000 scans, centroid mode). In a similar experiment, 0.5 μL of 160 nM myoglobin solution was introduced from the tip and then analyzed at a 20 nL/min flowrate using preset instrumental parameters. The averaged spectrum for the 20 min analysis showed a charge state distribution from 19+ to 42+ and from 11+ to 24+ for the carbonic anhydrase and myoglobin analytes, respectively (Figure 3B,C). Comparable results were also obtained with cytochrome *c* and lysozyme analytes with a loading of 1 μL or less sample solution. No problems were encountered because of gas bubbles (e.g., spray termination). The filling solvent does not significantly dilute the sample solution, because it is stored in only the frontal portion of the capillary and diffusion between the two zones is minimal. When sufficient solvent is first stored in the syringe, after each μL-size sample analysis, the capillary can be rinsed with a few μL solvent by fast forwarding the syringe pump, before loading another sample. We have sequentially analyzed up to four different samples with one filling of the solvent using the same capillary without significant cross contamination.

Conclusions

A simple nanoelectrospray arrangement is described that uses pulled capillaries having large tip inner diam-

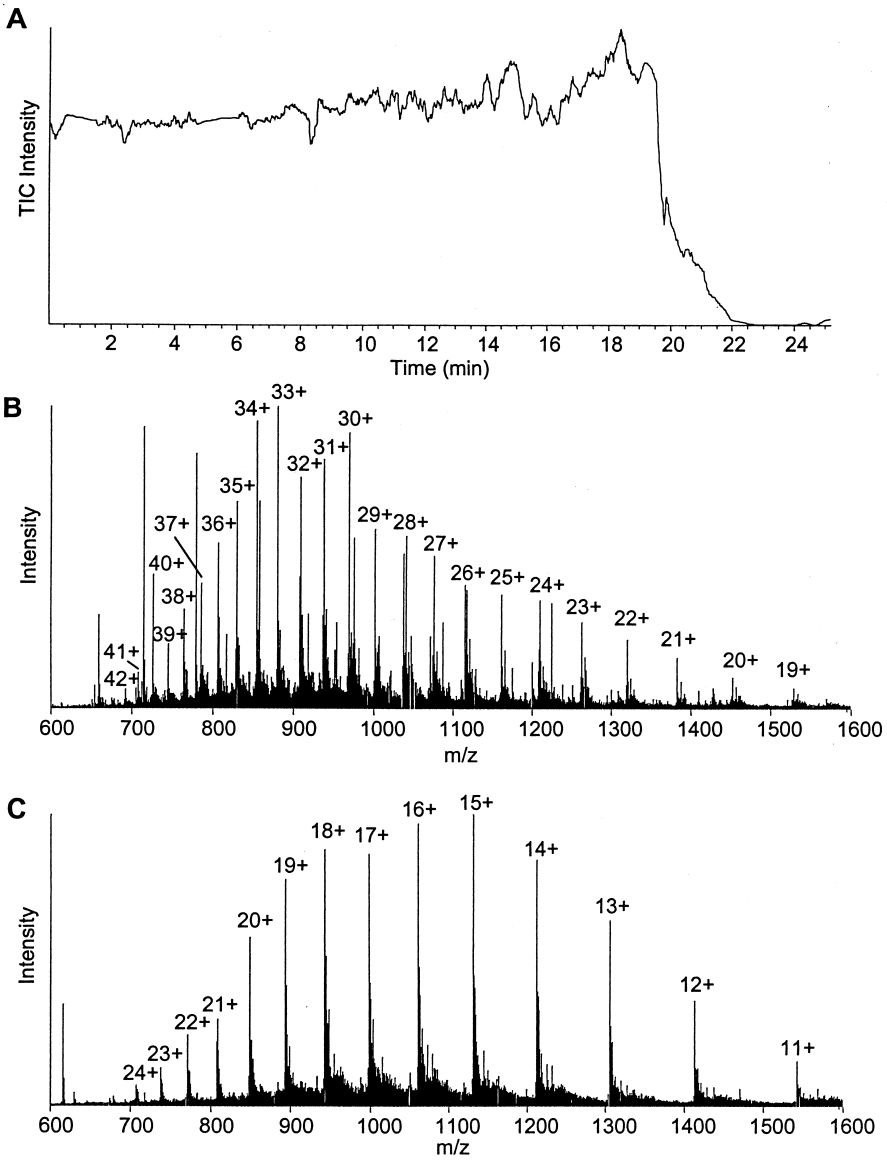

Figure 3. Microliter and submicroliter sample analysis. (**A**) Total ion current (TIC) and (**B**) averaged mass spectrum (20 min) for the analysis of 1 μL, 130 nM carbonic anhydrase (meas. MW. 29,020 $\pm$ 3 Da) solution at 50 nL/min flowrate. (**C**) Averaged mass spectrum (20 min) for the analysis of 0.5 μL, 160 nM myoglobin (meas. MW. 16,954 $\pm$ 2 Da) solution at 20 nL/min flowrate.

eters and without external metal coatings. A feature of this arrangement is reduced sample clogging. Sample infusion with a conventional syringe pump, operated at nanoliter-per-minute flowrates, produced stable signals and eliminated spray termination due to gas-bubble formation. This arrangement allows experiments to be performed under well-defined direct infusion flowrates. For standard protein samples, subattomole detection limits were achieved using an LCQ ion trap mass spectrometer. By drawing sample solution directly into the tip of the pulled capillary, submicroliter amount of sample can be handled and then analyzed for over 20 min at low flowrates. This methodology overcomes certain operational challenges that are associated with metal-coated emitters of much smaller i.e. tips, while maintaining comparable analytical capabilities.

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