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Perspective

A Personal Perspective on the Technological and Commercial Development of Nanospray and Nanobore LC-MS

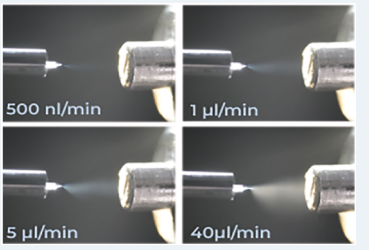
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ABSTRACT: Flow-rate-minimized electrospray ionization, commonly referred to as nano-electrospray or, more simply, nanospray, has evolved into an essential foundational technology in the development and proliferation of mass spectrometric-based proteomic analysis. Historically, nanospray was an offline technique, termed herein as static nanospray. Static nanospray paired well with the leading protein purification method of the day: two-dimensional polyacrylamide gel electrophoresis (PAGE). The demands of analyzing ever more complex samples have evolved rapidly with the introduction of LC-based methodologies. The introduction of shotgun proteomics technology, taking inspiration from the development of shotgun-based genomic sequencing, enabled the analysis of whole-cell protein digests in a single LC-MS run. As mass spectrometry continued to improve in both sensitivity and speed over the decades, concomitant advances in nanobore LC column technology and emitter performance have occurred. Over the past 30 years, we have been fortunate to see sensitivity gains progress from isolated PAGE gel bands to single-cell proteomics. Here, I offer a personal perspective on the evolution of nanospray and nanobore LC-MS technology and on the journey of bringing these tools from the bench to the global scientific community through commercialization.



INTRODUCTION

It is a pleasure to contribute this personal perspective to this special issue of *J. Am. Soc. Mass Spec.* honoring the career and contributions of Prof. Jack Henion to the field of mass spectrometry. Never expecting to have a career in mass spectrometry myself, I find it humbling that New Objective, Inc. is approaching its 30th corporate anniversary in January 2027. During graduate school in the Chemistry Department of Cornell University in the early 90s, I was well aware of the contributions being made to analytical chemistry and mass spectrometry through the efforts of multiple research groups: Prof. George H. Morrison (elemental ion microscopy), Prof. Fred W. McLafferty (organic mass spectrometry), Prof. Tom Brenna (isotope ratio mass spectrometry), and Prof. Jack Henion, whose pioneering work in quantitative LC-MS/MS would go on to shape an entire generation of pharmaceutical bioanalysis and, later, the commercial nanospray landscape through his founding of Advion. As a student, I did some small research projects using ion microscopy (dynamic secondary ion mass spectrometry) in the Morrison group,[1] but did not pursue MS technology development in any significant way. During the question-and-answer period of my oral thesis defense, Professor Fred W. McLafferty (1923−2021), a committee member, offered me a postdoctoral position in his lab. This was quite a surprise! Given that it was the Q&A period, the degree was not yet conferred, so I was obliged to answer "Yes!". Prior to this, my undergraduate and graduate studies had focused on the development and application of chemical light microscopy,[2,3] under the direction of Dr. Walter C. McCrone (1916−2002), McCrone Research Institute, then continuing through PhD studies with Professor George H. Morrison (1921−2004). Given the competition for positions in the McLafferty lab, when asked why he would want a microscopist on his team, he simply replied that he thought I was a capable scientist and would certainly make meaningful contributions.

Within the first week of joining Fred's group, I found a paper on my desk with a short note: "Gary, thought you might find this interesting—Fred." A quick skim of the paper got me very excited. It was the first publication by Wilm and Mann[4] on the theoretical underpinnings of electrospray ionization (ESI) and how it scaled down in flow rate, along with the first practical implementation of low-flow ESI for peptide analysis. Exceptional science always inspires! Later that week, I suggested to Fred that we would be in a strong position to participate in

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further technology development, since the Morrison group's instrumentation in near-field scanning optical microscopy (NSOM) using tapered optical fibers[5] could be readily adapted to mass spectrometry and nanospray—we would work toward substituting fused-silica tubing for optical fiber. Fred approved, with George's mutual support for continued use of their lab apparatus, and the nanospray research program in the McLafferty group was off and running.

STATIC NANOSPRAY

The seminal paper by Wilm and Mann,[4] which begins as a theoretical treatise on cone-jet formation in electrospray (Figure 1), provides a fundamental model of the scaling laws inherent to Taylor cone-jet formation and the Rayleigh instability of jet-to-droplet breakup. Their paper concluded with a brief experimental section showing a unique implementation of this scaling property: loading a few microliters of sample into a pulled glass capillary having an orifice dimension on the order of 1 to 2 μm. By carefully adjusting the applied spray voltage to a metal coating on the glass capillary outer surface, the electrostatic field was sufficient to draw the liquid out of the orifice, form a Taylor cone jet, and break up into a fine mist of droplets at a flow rate below 50 nL/min. Figure 2 shows an example of this method in action. This breakthrough technology enabled mass spectrometers of the day to thoroughly interrogate MS1 molecular ions and their fragment MS2 ions over a wide mass range, due to the multiple charges inherent to the ESI of peptides. De novo and database sequencing of peptides was now readily possible.[6] Samples limited to a few microliters could be directly and fully interrogated over the course of an hour or so of combined MS and MS/MS acquisition time.

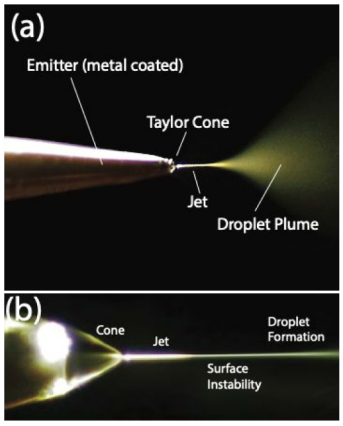


Figure 1. Dark-field photomicrographs of the canonical cone-jet electrospray mode. (a) A 30 μm diameter, metal-coated, fused-silica emitter was held at a voltage of approximately 2 kV, applied to the coating, relative to a counter electrode (not visible, positioned to the right side). Flow rate was 300 nL/min, with a mobile phase composition of 75% MeOH and 2% acetic acid. Labeled parts of the cone-jet morphology are shown (10x objective)×. (b) High-magnification (40×) view of the cone, jet, and droplet formation regions.

It is interesting to note that an early investigation by Zeleny,[7] one of the first to quantitatively investigate electrostatic breakup of droplets, introduced an experimental methodology strikingly similar to the micrometer-sized pulled-glass capillaries used in static nanospray. Zeleny also first characterized different electrospray modes, which are discussed later in this manuscript.

Initially, the McLafferty group's focus was to translate the glass capillary approach to fused-silica tubing.[8] The controlled dimensional tolerances and purity of fused-silica tubing meant that a highly optimized version of nanospray was possible, operable over a wide range of controllable flow rates, with extended operation below 1 nanoliter per minute. High-quality fused-silica tubing, having an inside diameter of 5 to 20 μm, combined with novel laser-pulling technology, was used to control emitter size from 1 to 20 μm. With sample fed to the silica emitter by capillary action from a pipet tip, samples could be readily ionized, and pipet tips could be swapped out rapidly. A single emitter could analyze multiple samples. As in the application of glass emitters, the only driver for flow was the electrostatic pull on the liquid at the end of the spray nozzle. No pumping or back pressure was required. Figure 3 shows some key elements of the instrumentation used in emitter fabrication and early experimental operation of fused-silica emitters. McLafferty referred to this low nanoliter-per-minute mode of operation as Picospray, which New Objective, Inc., would later

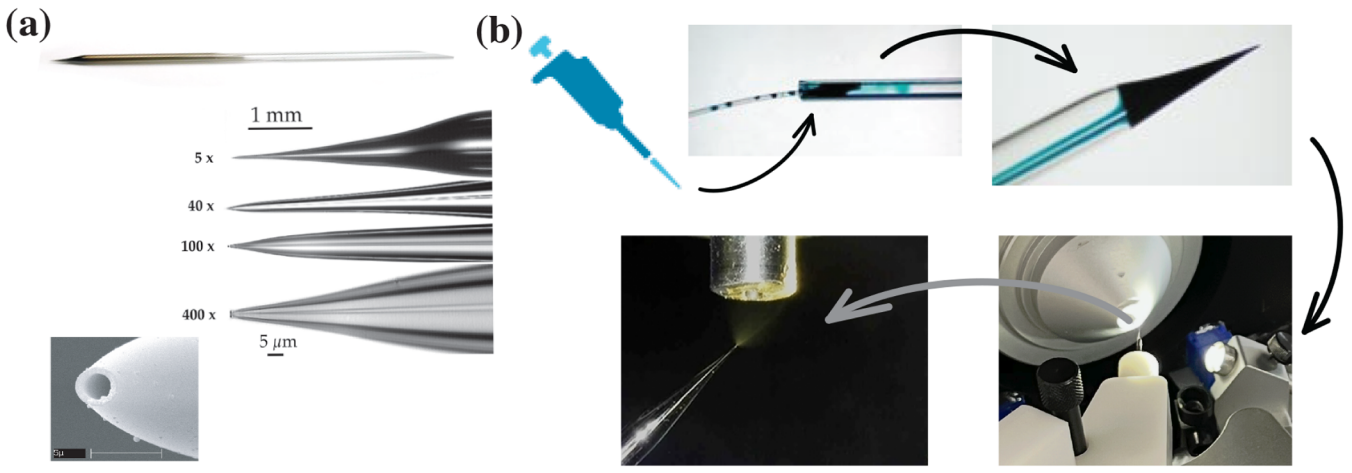


Figure 2. Static nanospray using pulled glass capillary emitters. (a) Typical appearance of a 5 cm long, metal-coated glass emitter, having an orifice inner diameter of 2 μm. (b) Static nanospray workflow: One loads a sample (1−10 μL) into the distal end of the emitter. Liquid is carried to the taper region and orifice by capillary action. After loading into the nanospray source, an electrical potential is applied between the capillary and the MS inlet, resulting in a low-volume droplet plume.

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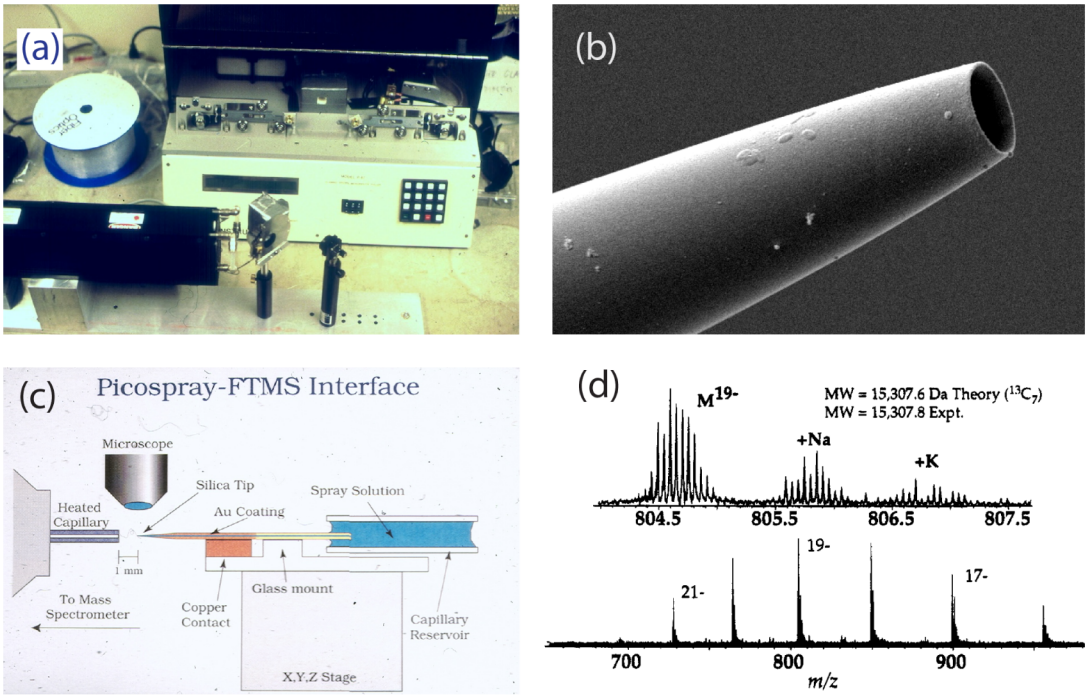


Figure 3. Translating static nanospray to fused-silica tubing. (a) In-lab-developed laser puller, likely the third such puller, built to handle optical fiber and fused-silica tubing.[5] (b) Typical 5 μm fused-silica emitter made from 150 μm OD × 20 μm ID fused silica; 2000× scanning electron microscope image. (c) Copy of group meeting slide presented to the McLafferty group circa March 1994. (d) Static nanospray data of a synthetic 50-mer DNA reproduced with permission from ref[8]. Copyright 1995 American Chemical Society.

license as patented technology[9] from Cornell University. The transition to fused-silica tubing would, of course, enable downstream integration of online capillary separation techniques, such as capillary electrophoresis and packed-bed (reverse-phase) liquid chromatography.

The defining characteristic of static nanospray is that the generation of liquid flow within the emitter is due solely to the electrostatic attraction of the liquid at the end of the emitter toward the counter-electrode (MS inlet). The electrostatic mechanism that generates the cone-jet spray results in subsequent hydrodynamic flow within the static nanospray emitter by capillary action. The cone-jet is the engine that pulls the train. From an operational standpoint, the higher the applied voltage, the higher the resulting flow rate. Unlike online (dynamic) nanospray, in practical use, the applied voltage is used to "dial in" the flow rate. Unfortunately, knowledge of the absolute flow rate requires careful calibration of voltage vs flow for a given emitter/inlet combination of a given fixed mobile phase composition. The exact flow rate depends on the emitter geometry (taper, ID, length), mobile phase composition, emitter-to-inlet distance, and MS source inlet design.

Smaller emitter inside diameter places two important constraints on the voltage vs flow relationship.[8] First, a smaller-diameter emitter improves spray stability at lower flow rates. A lower flow rate demands a smaller diameter at the base of the Taylor cone; thus, a smaller emitter provides a better flow-rate match. Second, a smaller-diameter emitter places an upper constraint on the flow rate due to the limit of mobile-phase viscous flow within the emitter's taper region.[9] Li and Cole[10] studied the impact of emitter diameter on charge-state distributions for multiply charged proteins and peptides. They observed a trend toward higher charge states when using smaller (1 μm) emitters. This shift is likely due to the smaller droplet size generated at lower flow rates supported by the smaller emitter. More recently, a number of groups have published[11−14] results on nanometer-scale ID emitters for intact and native protein analysis, where effective flow rates are vanishingly small ($\ll$1 nL/min). When parameters such as position, mobile phase, and applied voltage are carefully controlled, a Taylor cone-jet supporting a low flow rate can also be established with a relatively large emitter.[15] Both theoretical[4,16] and empirical observations, as discussed in the Tenets section, show that the diameter of the jet increases with flow rate. Decrease in jet diameter minimizes initial droplet size forming from capillary instability along the jet surface as flow rate decreases.

CAPILLARY ELECTROPHORESIS−MASS SPECTROMETRY: THE TRANSITION TO ONLINE SEPARATION

Having demonstrated the ability to achieve stable, efficient electrospray at ultralow flow rates compatible with the highly efficient 6T Fourier transform mass spectrometer (FTMS) in the McLafferty laboratory,[17] the new lab challenge was to push the boundary of sensitivity from a total-material-injection standpoint. If nanospray could generate meaningful ion current at a flow rate of 1 nL/min, then a specialized injection strategy could be developed to match low-flow performance metrics. Given the experimental simplicity and compatibility with fused-silica tubing, capillary electrophoresis (CE) was chosen as a primary methodology for integration of online separations with silica-emitter nanospray.

Ready availability of small-bore precision fused-silica tubing was the technological cornerstone for innovation. Five- to twenty-micrometer ID columns, having integral emitters of 2−5 μm, were coated on the emitter end with a multilayer gold/silicon dioxide coating.[18] Coating of the inner surface with an amino-silane directed electroosmotic flow from the distal inlet of

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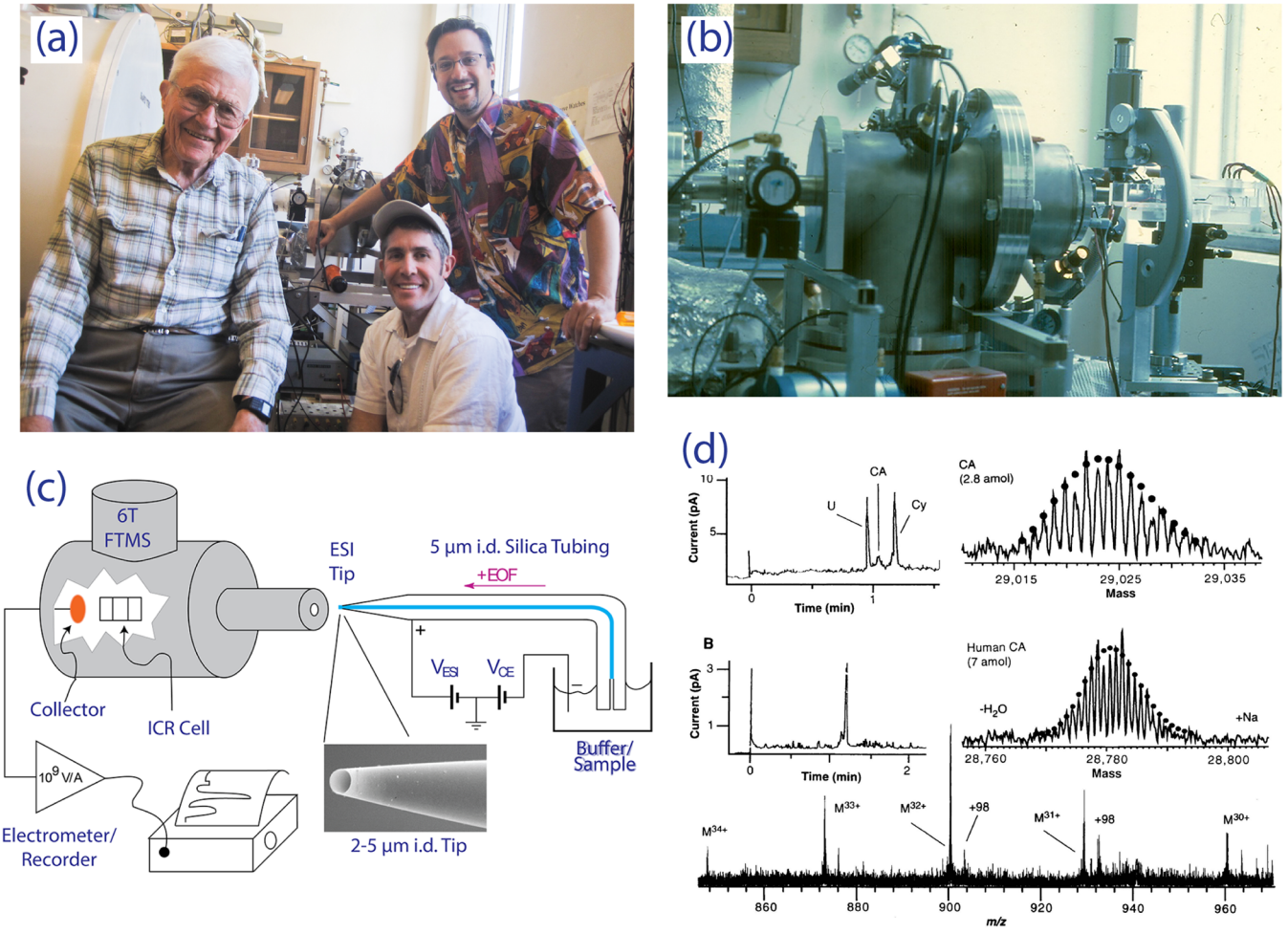


Figure 4. Transition to online nanospray. (a) Prof. Fred W. McLafferty, Prof. Neil Kelleher, and the author next to the legendary 6.2T FTMS, taken in 2011. (b) The homemade CE-MS system at the instrument inlet.[30] (c) Schematic of the CE-MS direct-flow sheathless CE-MS capillary setup. Column ID was 5 μm with a 2 μm ID emitter. Positive electroosmotic flow (EOF) was used in the nanoliter-per-minute range to supply continuous mobile phase for ESI. (d) Electropherogram and associated MS data for intact proteins injected and delivered to the instrument at the low attomole level, an early demonstration that nano-ESI-MS would be sufficient for single-cell proteomics. Reproduced with permission from ref 20. Copyright 1996 Association for Advancement of Science.

the column toward the emitter. By adjusting the potential at the emitter coating, along with an independent relative potential at the column's distal end, resulting electroosmotic flow ensured a stable, tunable flow rate for nanospray ionization. With accurate, bias-corrected electrokinetic injection,[19] small quantities of protein mixtures—equivalent to those present at the single-cell level—were readily ionized and detected with the 6T FTMS system.[20] The core McLafferty group team, comprising (now) Prof. Neil Kelleher, myself, and McLafferty, along with the experimental schematic and significant CE-MS results, is shown in Figure 4. Looking back on this work with satisfaction, we believed then that the demonstration of single-cell protein sensitivity would spur ongoing research. It is gratifying to see that single-cell proteomics is now a widespread activity in proteomic research.[21−23]

NEW OBJECTIVE, INC.: COMMERCIALIZATION—BRINGING ONLINE NANOSPRAY TECHNOLOGY TO MARKET

After the publication of the Science paper,[20] numerous colleagues and a few established MS vendors expressed interest in the emitters and CE columns being lab-fabricated. At that time, I was fully committed to an academic career. However, after a series of rather intense conversations with Fred, it was first clear to him and then ultimately to me that staying in academia was not my true desire. After a few sleepless nights and much discussion with my spouse, Emily Ehrenfeld, we decided that we would take the fused-silica emitter and column technology to market, working to develop a viable consumable business. Fred had been quite supportive that we apply for patent(s) through the university to cover the technology. On this basis, we set off to Cambridge, Massachusetts, to found New Objective, Inc., in early fall 1996. New Objective, Inc., would then license Cornell Intellectual property[9] in an exclusive relationship.

ADVION, INC.: COMMERCIALIZATION—CHIP-BASED STATIC NANOSPRAY

As the fused-silica nanospray emitter technology was moving forward in the McLafferty group, ultimately toward commercialization, the R&D team from Advion, Inc., a company cofounded by Cornell Veterinary School Prof. Jack Henion, was working at Cornell's National Nanofabrication Facility to prototype a concept for a chip-based static nanospray emitter

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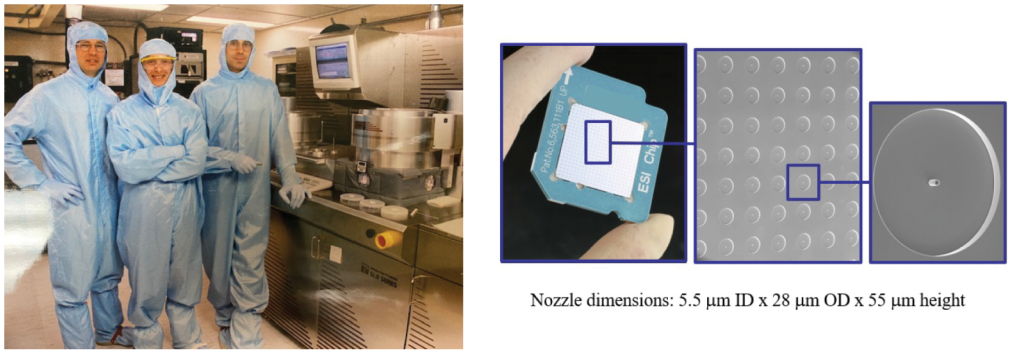


Figure 5. (a) Chip nanospray team (Gary Shultz, Simon Prosser, Tomas Corso) from Advion Inc. (b) Static nanospray chip commercialized by Advion. Photos courtesy of Prof. Jack Henion (Emeritus), Cornell University; used with permission.

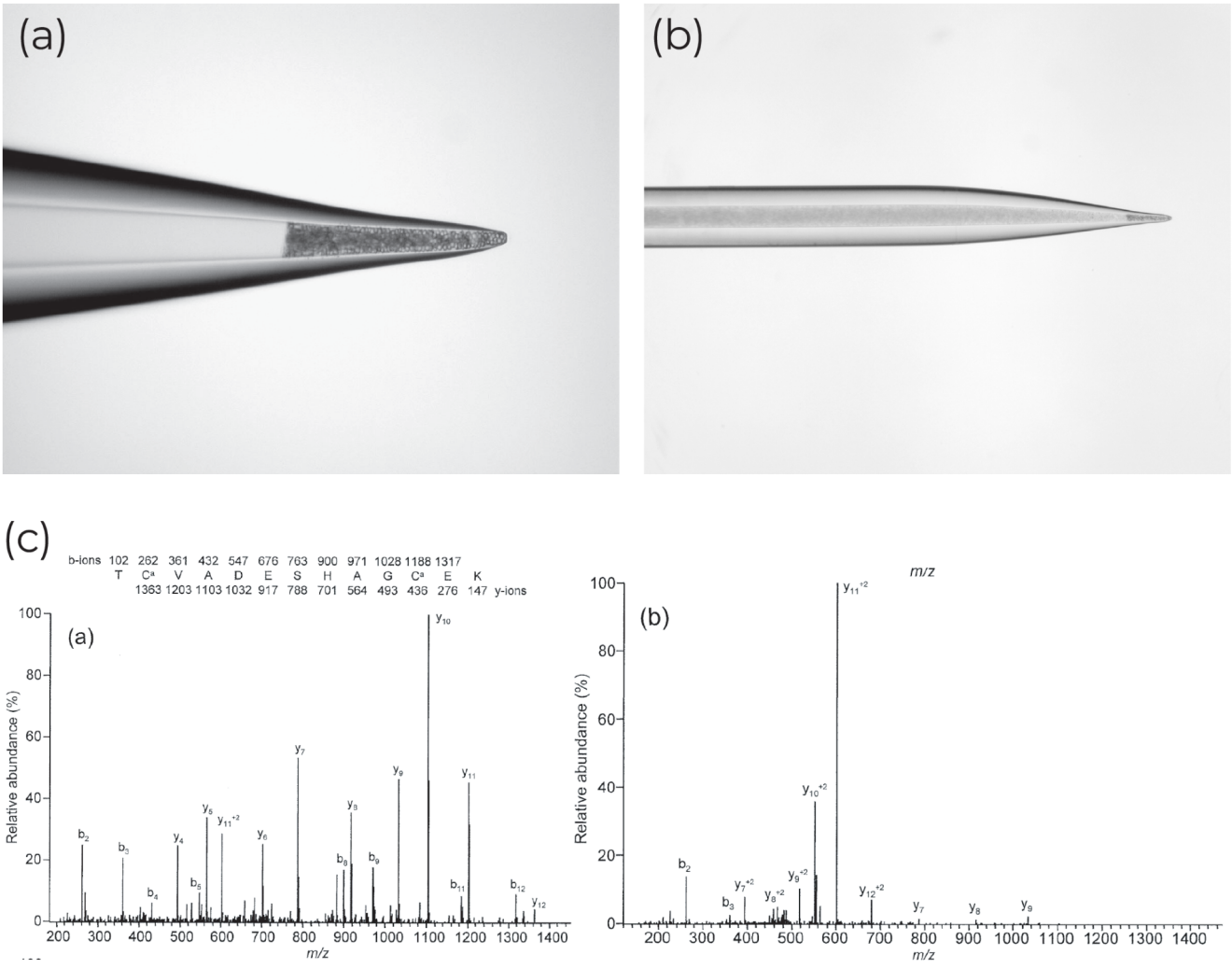


Figure 6. (a) Early example of the self-packed PicoFrit emitter, 75 μm ID column × 15 μm ID tip. The frit is characterized by a low added back pressure and is stable enough for packing UHPLC media at pressures >1400 bar. (b) A 75 μm ID PicoFrit column packed with 1.9 μm C18 reversed-phase media. (c) The author believes this to be the first spectra in JASMS to use a self-packed PicoFrit column, used here for sequencing tryptic peptides from BSA. Reproduced with permission from ref 43. Copyright 2001 American Chemical Society.

array.[24] A 10 × 10 square planar array of 5 μm ID nozzles was ultimately commercialized as the Advion NanoMate system. The NanoMate system combined autosampler functionality with pipet-driven sample delivery to the back side of each silicon nanospray emitter. Key advantages of the system are the absolute reproducibility of emitter geometry and, perhaps most significantly, a nonredundant fluid path resulting in zero carryover between samples. Later revisions of the system enabled online functionality for the incorporation of conventional nanobore LC columns.[25] The Advion chip development team and a photo of an early ESI chip are shown in Figure 5. The direct competition emerging from the other side of the Cornell campus under Advion's direction, as well as the already established European company Protana Co., Odense, Denmark

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(led by Ole Vorm working with Mathais Mann, later renamed Proxeon A/S), spurred us on to continue innovation in online nanospray. Advion and Proxeon were, at the time, both focused heavily on offline, static nanospray. With clear commercial interest in more sophisticated nanospray products, friendly competition was driving innovation in a (then niche) area of mass spectrometry—one that was poised to grow.

It is appropriate to note here an added benefit to ultralow flow delivered by static nanospray is an increase in the surface area per unit volume of mobile phase. Models established in ESI mechanistic theory[26−28] propose that as ions transition from liquid to gas phase, solvated ions transition from the bulk to the excess surface charge on the droplet perimeter. By minimizing droplet size while increasing total surface area per unit volume, one maximizes the probability that an analyte can reach that excess surface charge. Furthermore, solvent evaporation cycles are minimized prior to ionization events. Karas and coworkers had noted[29,30] that a decrease in flow rate and droplet size increases ionization signal of specific analytes in a mixture, reflected as a decrease in ion suppression. This effect was further explored in collaboration between Dr. Jing-Tao Wu and New Objective to see if nanospray could be exploited as a "universal response" ionization technique.[31−33] It was shown that within a defined chemical space, ionization trended toward normalized concentration response as through-emitter flow rate decreased.

Back on the commercial front, as New Objective began manufacturing static and dynamic nanospray emitters in January 1997, it became clear that capillary electrophoresis and the associated electrophoretic capillary techniques were in commercial decline.[34] One-by-one, commercialized CE instruments fell to the wayside, leaving only one or two vendors with sustained commercial interest. As CE-MS continued in academic research, there was a parallel track of interest in combining reverse-phase LC with nanospray. Richard Caprioli and the team led by Emmett and Caprioli,[35] as well as the long-established Hunt group,[36] had been using packed C18 fused-silica columns (75 μm ID) for highly efficient de novo sequencing of tryptic protein digests. Furthermore, as the reality of the Human Genome Project was drawing to draft consensus, smaller organisms had already had their genomes sequenced. It was clear that (partial) peptide sequence information would enable complex mixture protein ID at the organism level through shot-gun sequencing approaches.[37,38]

With the commercialization of standardized silica spray emitters, New Objective focused attention on integrated (reverse-phase HPLC) emitter-column development. Customer feedback from those self-packing standard (unfritted) fused-silica emitters was clear: although one could pack an emitter-column without a frit, having an appropriate outlet frit would offer advantages of reproducibility, flow-rate compatibility, robustness, and spray stability. The common practice for frit fabrication at the time was the so-called Kasil methodology,[39] in which a silicate frit would be fabricated in situ from a potassium silicate solution with addition of an organic cosolvent porogen. Unfortunately, Kasil frits, while readily fabricated, yielded limited column-to-column reproducibility. Frit back pressure was high, impeding fast and efficient packing of chromatographic columns, especially given the low packing pressures (<100 bar) in common use at the time.[40]

Development of a low-back-pressure frit followed, resulting in a key international patent for New Objective after an insightful series of observations by New Objective's cofounder Emily Ehrenfeld.[41,42] In those early days, Emily was often "pulled away" from essential business activities to work in the manufacturing lab. The resulting packed-emitter column, having an integral, low back-pressure frit, is shown in Figure 6. By 2010, thousands of laboratories were using self-packed[43] or prepacked PicoFrit columns for in-depth proteomics work. Nearly 25 years later, self-packed PicoFrit columns still make up a considerable number of additional annual publications to New Objective's citation portfolio.

A fritted nanospray column has numerous manufacturing and operational advantages.[44] The frit provides a reproducible bed for column packing using slurry methods at either low or high pressure. As a result, such columns provide reproducibility of their pressure-to-flow profiles. The chromatographic peak shape is also maintained as the frit, positioned at the very end of the emitter, ensures that peaks elute directly from the packed LC bed to the emitter orifice. Fritted emitters also support spray stability: frit placement at the emitter orifice ensures no sudden pressure drop ahead of the orifice, reducing mobile-phase outgassing between column and emitter orifice common to sudden pressure drops. Gas bubble formation can be especially notable when connecting a separate column to an emitter, along with the tell-tale periodic stop-start of ion current as bubbles pass through the emitter. Finally, fritted emitters enable a wide range of packing materials, whether by the base composition, size, or surface chemistry.

INDUSTRY AND COMMUNITY OBSERVATIONS: THE TENETS OF NANOSPRAY

After the development of glass and fused silica emitters, an extended period of incredible scientific and engineering creativity followed. For many months between 1998 and 2010 or so, it seemed like every issue of *Analytical Chemistry* contained a novel way to do some variation of nanospray ionization. Implementations ranged from a beautifully simple, conically rolled piece of polyimide tape[45] to highly integrated microfluidic systems.[46−48] All manner of substrates have been investigated: (noncapillary) glass,[49] silicon,[50] paper,[51] and polymers[52,53] to name a few. Various geometries have been explored: pipet tips,[15,52] tiny holes in chips,[54,55] pointed rods,[56] diving-board style,[57] inspiration from rolling-ball,[58] and fountain pens[59] to name a few. Of course, one cannot skip the various multinozzle approaches,[60−63] adding to the innovation.

A core conclusion one can draw from this experimental variety is that only two requirements are needed to generate nanospray of an appropriate (ESI compatible) mobile phase: (1) a sufficiently small, defined area to support the liquid for cone-jet formation, and (2) a sufficiently high electric field gradient between the liquid and a neighboring counter-electrode to yield cone-jet formation at that defined area. Requirement (2) implies that some form of electrical contact be made with the liquid.

Figure 7 demonstrates direct observation that droplets (not visible directly using conventional cameras and transmitted-light illumination) emerge from the cylindrical, rod-like jet forming at the apex of the Taylor cone, defined here as the "liquid tip" (i.e., droplets do not emerge from the emitter directly). Here, we show a fixed fused-silica emitter geometry (10 μm ID) positioned 2 mm from a flat steel plate counter-electrode located 2 mm outside the field of view to the left. Without any other experimental variation, changing the mobile phase flow rate changes the diameter of the jet emerging from the Taylor cone. This behavior exactly follows the theoretical model developed by Wilm and Mann.[4] Lower flow rates yield a smaller-diameter jet, resulting in smaller initial droplets in the plume.

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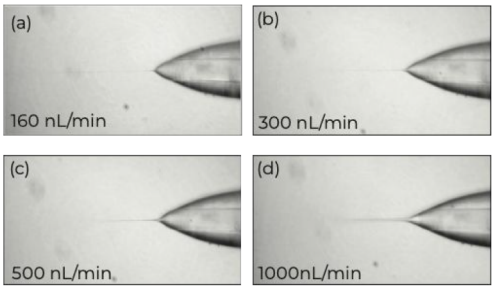


Figure 7. Droplet-jet emission from the Taylor cone apex ("liquid tip") at four different flow rates. Transmitted light photomicrographs (40× objective) of a 10 μm SilicaTip, supplied with 50% ACN and 0.1% formic acid from a constant-flow-rate pump. Spray voltage (optimized for each flow rate for a stable cone-jet) was applied to a platinum wire junction contact between pump and emitter. The counter electrode is outside the field of view. Note that, with progression from (a) 160 nL/min, (b) 300 nL/min, (c) 500 nL/min to (d) 1000 nL/min, the diameter of the jet emerging from the Taylor cone increases. Subsequent breakup of the jet into individual droplets (not visible) occurs to the left of the jet. The observed increase in the blurry edge of the jet is a result of the fast droplet-formation process taking place.

Clearly, the geometry of the emitter, and therefore any Taylor-cone supporting surface, plays what one could call a secondary role in the physical formation of the ion-generating droplet plume.

Drawing on three decades of practical development in our laboratory, together with broader customer observations and electrospray literature, I propose the following tenets as a concise framework for understanding the physical aspects of nanospray ionization. Not to be considered formal axioms, but perhaps as working principles to guide development—and that I believe remain useful as the field continues to push toward a wider range of flow rates, smaller sample volumes, single-cell sensitivity, and clinical applications.

TENETS OF NANOSPRAY

1. **Droplets emit from the Taylor cone-jet, not from the physical emitter.** The electric field at the Taylor cone terminus can be orders of magnitude higher than at the nozzle surface.[4] The emitter's role is simply to provide a stable anchor for cone formation, whether the cone is steady or oscillatory. The supporting surface in contact with the base of the Taylor cone, defining the emitter area, need not be a cylindrical orifice.
2. **Static nanospray—in which zero emitter-to-inlet potential corresponds to nominally zero flow—demonstrates that flow rate is primarily governed by the electric field gradient generated by applied potential, together with the area supporting the Taylor cone base; not directly by emitter ID.** Applied potential and droplet size are directly related under these so-called static conditions: lower voltage yields smaller droplets for a given geometry and mobile phase composition. There does not appear to be a measurable lower limit of the flow rate required for ionization in static nanospray methods.
3. **Initial droplet size is a function of mobile phase (mass) flow rate, not nozzle diameter**. Emitter geometry (taper, diameter) is chosen to optimize spray stability at the target flow rate.
4. **High voltage can be applied via a conductive emitter, a conductive coating, or a liquid-junction electrode.** Remote liquid-junction contact typically requires a higher applied potential for stable spray, reflecting the voltage (current × resistance − iR) drop between electrode and emitter.
5. **The small nanospray plume enables high collection efficiency (transmission) at API-MS inlets.** The vacuum suction at the inlet can capture up to 100% of a droplet plume, as the physical size of the plume is milliliter scale and the total solvent load on the inlet vacuum stage is low.
6. **A stable cone-jet is one of many possible spray modes.** Most spray modes produce useful ion current for mass spectrometry. Oscillatory modes are most common with pumped flow (LC or syringe pump) to the electrospray emitter, with characteristic frequencies spanning 0.1 Hz to >100 kHz.[64]
7. **Smaller primary droplets maximize the mobile phase surface area per unit volume.** Fewer droplet fission cycles are required before gas-phase ion formation; ion suppression is minimized under these conditions.

PATH TO INTEGRATED NANOLC-MS

The rapid and widespread growth of mass-spectrometry-based proteomics led to an explosion of laboratories adopting nanobore LC-MS technology. Effective application of nanobore LC during this time was, of course, technically challenging, requiring significant expertise and training. One had to manually integrate instruments from different vendors, become an expert at microplumbing, manage multiple sample preparation techniques, master effective column packing, learn manual flow-rate calibration, optimize LC method development, and learn both ESI and MS tuning—and the list goes on. These fundamental needs sparked considerable effort in both academic and commercial laboratories to push the integration of nano-LC, nanospray ionization, and mass spectrometry. While full HPLC-ESI source-MS integration (including, for example, integration of nano-LC pumps) has not achieved commercial viability, vendors have chosen to focus on integrating selected system elements. One consistent theme of these efforts (examples of which are shown in Figure 8) is to either reduce or eliminate the need for making multiple ultrahigh-pressure connections of suitable quality for nano-LC. This usually takes the form of a card-like microfluidic device, cartridge, monolithic assembly, or similar—containing the nanobore LC column, column thermal control, high-voltage contact, nanospray emitter, and positioning elements.

Agilent was the first to commercialize an integrated approach with the HPLC-Chip and associated HPLC-Chip Cube interface.[48,65] The key element of the HPLC-Chip system was the integration of a layered polyimide microfluidic device directly with an injection valve, sample trap, analytical column, and nanospray emitter. To this day, it marks the most highly integrated commercialized system for nano-LC/MS. Unfortunately, the HPLC-Chip did not incorporate column thermal control for elevated-temperature LC, and it predated the introduction of UHPLC media. The HPLC-Chip system was followed by introductions from other vendors.

Waters Corporation with the Trizaic system (subsequently developed as ionKey), and Thermo Fisher Scientific with the EASY-Spray column and source system,[66] which had been under development preacqusition by Thermo Fisher at the aforementioned Proxeon A/S. These two systems integrate the LC column, column thermal control, ESI voltage, and nanospray

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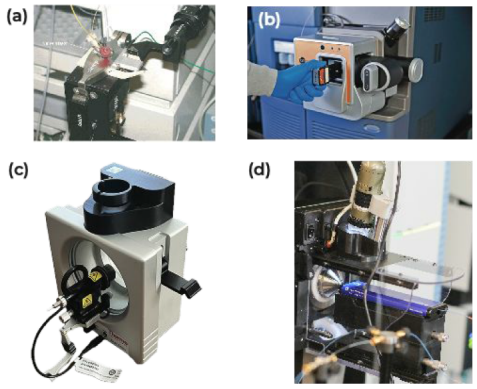


Figure 8. Examples of commercialized, integrated nano/micro-flow systems. (a) Agilent HPLC-Chip: a layered planar, polyimide-based column that integrated connections, injection loop, trap column, analytical column (75 μm ID), electrical contact, and nano-ES emitter. (b) Waters ionKey: a ceramic-molded chip assembly that integrated the capillary analytical column (150 or 300 μm ID), all fluidic connections, column temperature control, coupled to an emitter. Photo from *C&EN* 2017, 95 (10), 18−23. (c) Thermo Fisher Scientific EASY-Spray system: integrates an analytical column (75 or 150 μm ID), column temperature control, ESI high voltage, and nano-ES emitter into a single package that plugs into the EASY-Spray source housing. (d) New Objective Universal Flow Station source with an integrated nano-column emitter (PicoChip −75 μm ID)) or capillary-column emitter (FlowChip −150 μm ID) magnetic snap-in cartridges integrate analytical column, column temperature control, sheath-gas connection, and ESI high voltage. For (c) and (d), the only connection made by the end user is a single line coming from the UHPLC pump/autosampler. (a) Reproduced by permission from ref 48. Copyright 2005 American Chemical Society. (b) Reproduced by permission from ref 67. Copyright 2017 American Chemical Society.

emitter. In addition, the ionKey[67] provided all fluidic connections directly in the column cartridge, while the EASY-Spray requires only a single connection to the HPLC/autosampler. New Objective introduced its beta-test PicoChip product in 2011, which integrated nanocolumn, ESI voltage contact, and fritted nanospray emitter into a single cartridge design with a precisely precalibrated emitter position. The PicoChip design was later improved to incorporate column temperature control and the option of integral sheath gas.[68] The author believes that sheath gas, which is common in conventional ESI, plays a key role in transitional LC capillary and micro column formats, as discussed in the next section.

THE (NEAR) FUTURE: LARGER ID COLUMNS AND HIGHER FLOW RATES

Work in an increasing number of proteomics groups over the past few years has demonstrated the need and benefits of analyzing a greater number of samples per hour.[69−71] Once dominated by long columns running multihour gradients, our industry is shifting to shorter columns, faster gradients, and larger sample sets. While 75 μm ID continues to be the industry standard for packed nanocolumns, larger ID formats are gaining increased attention. The primary rationale is that the high sensitivity of current-generation MS systems is more than sufficient: larger ID capillary columns, 150 to 300 μm ID, provide real-world benefits in improved robustness and reduced injection cycle time, without sacrificing fit-for-purpose sensitivity.[72,73] Indeed, single-system throughput in the range of 1000 samples per day has been reported in the (non-peer reviewed) literature.[74]

The typical fused-silica emitter, with an ID of approximately 5 to 20 μm, is capable of generating meaningful ion current from less than 100 nL/min to well over 10 μL/min. However, spray stability typically suffers under these conditions, as spray mode typically deviates from the canonical cone-jet mode.[64] At flow rates above 1 μL/min or so, pulsing spray modes become more prevalent.[75] Pulsing spray modes inhibit MS/MS data collection in both data-dependent and data-independent acquisition schemes, as the spray is, in effect, turning on and off.[75,76] While high-frequency oscillation (>1 kHz) may not have a high impact on MS signal[77] lower-frequency (<100 Hz) phenomena

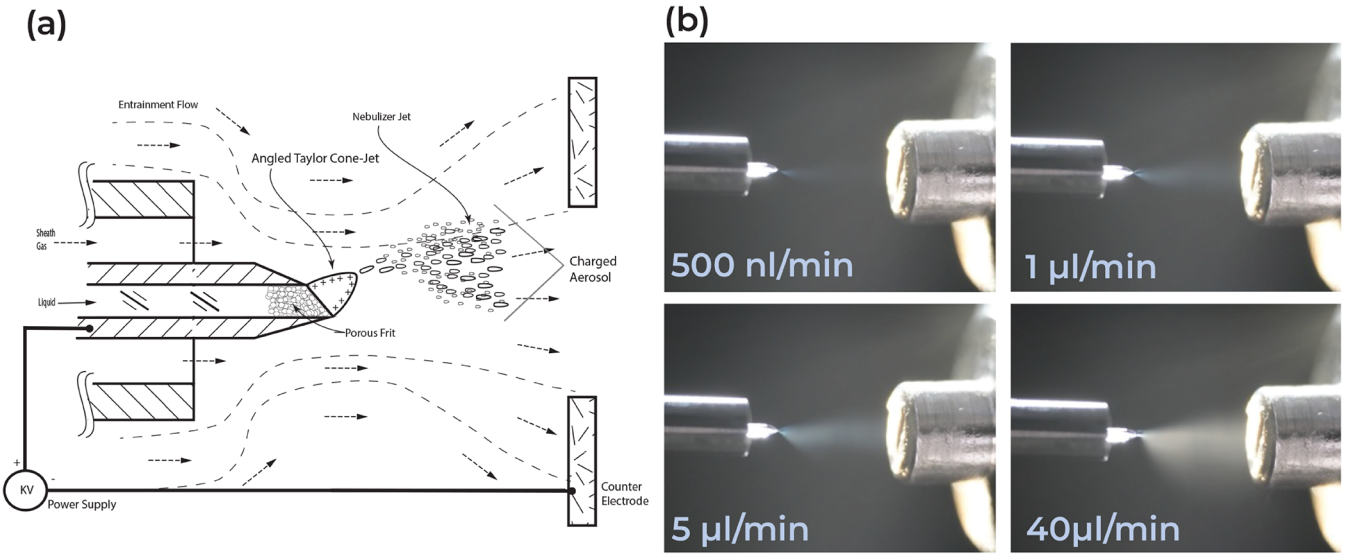


Figure 9. Novel design for a nano/micro-flow emitter as disclosed in US patent no. #12,211,684. (a) The dual-beveled, fritted emitter enables the combined advantages of nanoflow operation with high-flow-rate stability. The frit/bevel combination provides a base for redirecting fluid flow at an angle to incoming sheath gas, promoting asymmetric shear of the liquid as it forms a cone-jet. (b) Dark-field images of the spray plume at four different flow rates (indicated in each inset) for an ESI voltage of 3200 V and a sheath gas flow of 0.65 L/min (ca. 2.6 bar). Note the radial asymmetry of the resulting plume.

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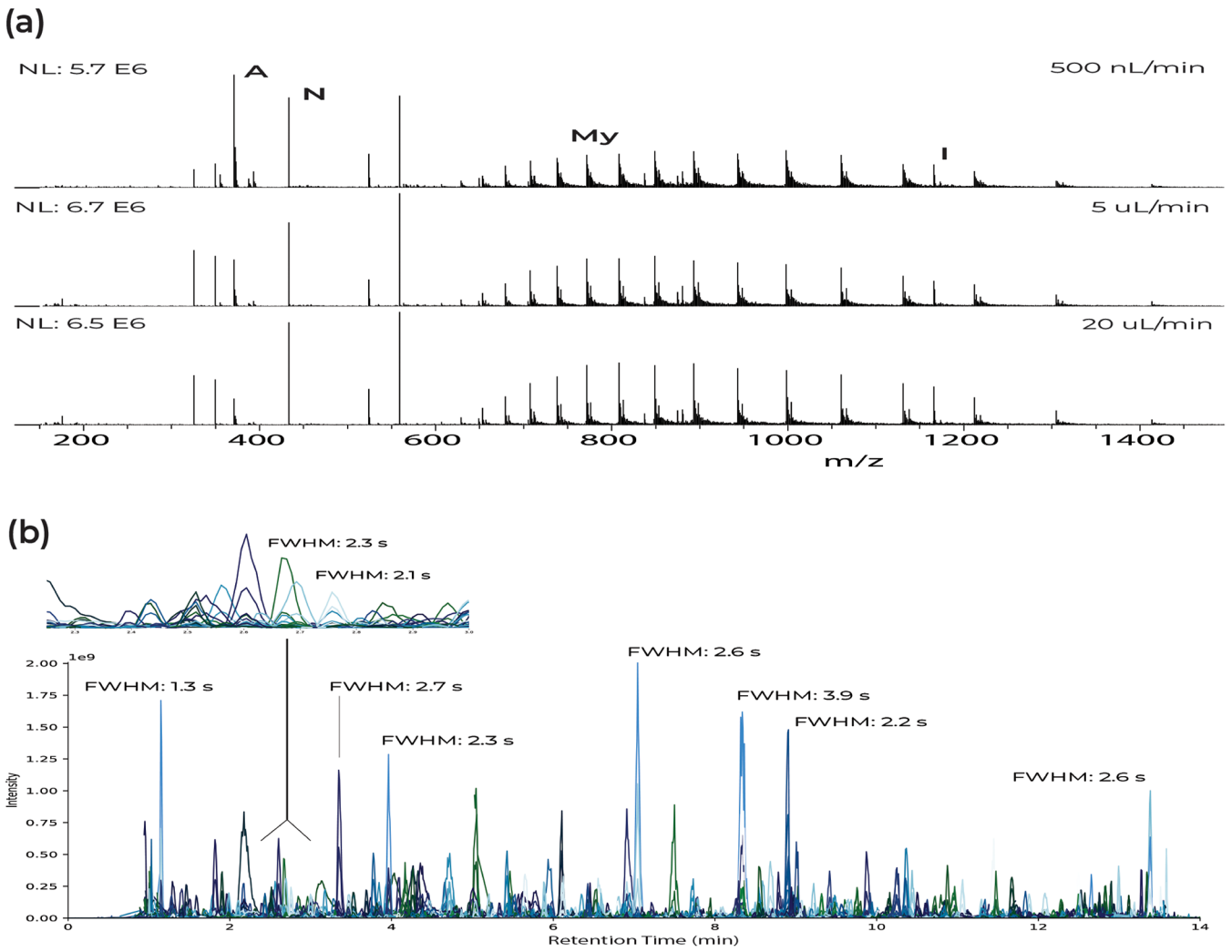


Figure 10. Infusion and chromatographic results of the dual-bevel, fritted emitter. (a) Infusion of a peptide/protein mixture (1 μM each component; A = angiotensin, N = neurotensin, I = insulin, My = myoglobin molecular ions) at three different flow rates (500 nL/min, 5 μL/min, and 20 μL/min) using the same position, voltage (2900 V), and gas settings.[78] (b) Extracted-ion chromatograms (discrete MS1 signals plotted, each LC peak is an individual m/z and charge-state value) for reverse-phase LC using a 150 μm ID column packed with 1.5 μm C18, terminated with the dual-bevel emitter. 15 min gradient to 35% ACN of Hamster-cell tryptic digest. The LC was a Thermo Fisher Neo Vanquish; MS a Thermo-Fischer Orbitrap Astral. Typical chromatographic peak width of 2−3 s. full width at half maximum (FWHM).

will.[64,75] A second factor in the employ of nanospray-sized emitters at higher-than-nanospray flow rates is limited emitter lifetime, expressed in either the number of samples injected and/or number of days of continuous operation. We have observed (unpublished data) that emitter lifetime is often related to both the total mobile-phase volume an emitter delivers and the number of samples injected, before spray quality degrades.

In response to the experimental needs driven by larger sample cohorts, demanding higher robustness and throughput, the author has recently developed a tapered emitter-column geometry featuring multiple beveled surfaces in combination with a frit at the emitter orifice.[78] The novel emitter design is shown schematically and operationally in Figures 8 and 9. The dual-bevel, fritted emitter design yields different spray surfaces and different Taylor cone directions depending on the volumetric flow rate through the emitter (Figure 9b). At true nanospray flow rates, the spray preferentially emits from the very end of a sharp secondary bevel. As the flow rate is increased—typically transitioning at 1 μL/min and above—the Taylor cone origin shifts to the surface of the off-axis frit. As a result, the direction of the initial cone-jet spray shifts off the emitter axis. Importantly, the base of the cone-jet region is now in a "clear" shear zone of the concurrently flowing coaxial sheath gas. This results (Figure 9a) in a stable, sheath-gas-supported cone-jet formation that has elements of both nanospray and conventional ESI plume formation. Note in Figure 9b the asymmetric nature of the plume, resulting from the off-axis Taylor cone base.

Some key operational aspects of this emitter: a wide flow range (100 nL/min to ≥40 μL/min); limited need to adjust spray voltage, with 2900−3200 V typically "just working"; a modest sheath-gas flow (0.6−0.7 L/min) that is highly beneficial for spray stability above 1 μL/min; and spray stability through rapidly changing mobile-phase gradients (Figure 10b). The emitter orifice is both fritted and elliptical in shape, greatly enhancing clog resistance. Finally, the emitter is physically robust, having demonstrated the ability to survive cleaning methods ranging from ultrasonic rinsing to direct mechanical cleaning, yielding extended emitter lifetime. This technology has been newly commercialized in New Objective's FlowChip product line.

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CONCLUSION

Nanospray ionization has matured from a laboratory validation of a theoretical framework—a pulled glass capillary loaded with a few microliters of sample—into a foundational technology underpinning modern proteomics; from high-throughput biological mass spectrometry to single-cell analysis. The path from Wilm and Mann's original static nanospray demonstration, through fused-silica emitters and CE-MS demonstrating single-cell sensitivity, to today's integrated nanobore LC-MS platforms reflects a three-decade evolution shaped by electrospray's scaling laws,[4,26,79] MS instrument performance improvements, the introduction of sub-2 μm LC particles, and the evolving demands of the life sciences. The tenets proposed in this Perspective are offered as a concise distillation of what has been learned along the way, and as a reference frame for researchers entering the field.

Looking forward, the continued shift toward shorter gradients, larger-ID capillary columns, and higher sample throughput is pushing nanospray into flow regimes that blur the historical boundary between nanospray and conventional ESI. Hybrid geometries such as the dual-bevel fritted emitter described herein suggest that the next generation of low-flow ionization will be defined less by absolute flow rate and more by the integration of tailored emitter design, sheath-gas dynamics, and column architecture (packing chemistry, ID, and length) into the mass spectrometers of today and the future. It has been a personal honor to be part of this dynamic research and instrumentation community, and to share my perspective in this issue of JASMS honoring one of our best, Prof. Jack Henion.

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Notes

The author declares the following competing financial interest(s): The author is the co-founder, and currently serves as Research Fellow at New Objective, Inc. and has clear financial conflicts of interest in nanospray LC-MS technology and related product commercialization.

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