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Analytical Properties of the Nanoelectrospray Ion Source

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The nanoelectrospray ion source (nanoES) has recently been developed and described theoretically. It is different from conventional electrospray sources and from other miniaturized electrospray sources by (i) its 1−2 μm spraying orifice achieved by pulling the spraying capillary to a fine tip, (ii) its very low flow rate of ~20 nL/min and the small size of droplets it generates, and (iii) the absence of solvent pumps and inlet valves. The fabrication and operation of nanoES needles is described in detail. Solutions with up to 0.1 M salt contents could be sprayed without sheath flow or pneumatic assist. Improved desolvation in nanoES led to instrument-limited resolution of the signals of a glycoprotein and the ability to signal average extensively allowed the C-terminal sequencing of a 40 kDa protein. Extensive mass spectrometric and tandem mass spectrometric investigation of the components of an unseparated peptide mixture was demonstrated by verification of 93% of the sequence of carbonic anhydrase. A rapid and robust desalting/concentration step coupled to the nanoES procedure allows the direct analysis of impure samples such as peptide mixtures extracted after *in-gel* digestion.

At the end of the 1960s Dole et al. described the idea that electrospraying a liquid containing analyte molecules might liberate these as ions and make them amenable to mass spectrometry.[1] Electrospray's potential as a mass spectrometric technique was first demonstrated in the early 1980s by Fenn et al. for small molecules[2] and in the late 1980s for proteins.[3] Independently and at about the same time, Russian groups achieved similar results on small molecules.[4] Although it was its capability to analyze intact proteins which sparked widespread interest in electrospray mass spectrometry (ESMS), the technique is now an indispensable tool in a wide range of applications of mass spectrometry and of protein science.[5−7]

Recently, we developed and experimentally verified a theoretical model for the electrostatic dispersion of liquid in electrospray which treats the spraying process in analogy with liquid metal ion sources.[8] The model predicts a proportionality between the two-thirds power of the flow rate and the size of droplets emitted from the tip of a stable Taylor cone. Small droplets have several desirable analytical properties; one of them is a high surface-to-volume ratio which makes a large proportion of analyte molecules available for desorption. The low flow would in itself be beneficial by providing a long measurement time at unchanged signal level, because of the concentration independence of the signal in electrospray.

The low flow was realized experimentally with gold-coated capillaries drawn with a short taper to a fine tip of ~1 μm inner diameter. About 1 μL of solvent is loaded directly into the capillaries; i.e., no solvent pumps or inlet valves are needed. The measured flow rate of these capillaries is only 20−40 nL/min and the predicted droplet diameter according to the above-mentioned equation is less than 200 nm, about 100−1000 smaller than the volume of the 1−2 μm droplets generated by conventional electrospray sources. At concentrations of 1 pmol/μL such droplets contain on average only one analyte molecule. This "nanoelectrospray (nanoES)" ion source has been the only electrospray source in the authors's laboratory for the last two

(1) Dole, M.; Mach, L. L.; Hines, R. L.; Mobley, R. C.; Ferguson, L. P.; Alice, M. B. *J. Chem. Phys.* **1968**, *49*, 2240−2249.
(2) Yamashita, M.; Fenn, J. B. *J. Phys. Chem.* **1984**, *88*, 4451−4459.
(3) Meng, C. K.; Mann, M.; Fenn, J. B. *Z. Phy. D: Atoms, Mol. Clusters* **1988**, *10*, 361−368.
(4) Alexandrov, M. L.; Gall, L. N.; Krasnov, N. V.; Nikolaev, V. I.; Panvlenko, V. A.; Shkurov, V. A.; Baram, G. I.; Grachev, M. A.; Knorre, V. D.; Kusner, Y. S. *Bioorg. Khim.* **1984**, *10*, 710−712 (in Russian).
(5) Fenn, J. B.; Mann, M.; Meng, C. K.; Wong, S. F.; Whitehouse, C. M. *Science* **1989**, *246*, 64−71.
(6) Smith, R. D.; Loo, J. A.; Edmonds, C. G.; Barinaga, C. J.; Udseth, H. R. *Anal. Chem.* **1990**, *62*, 882−899.
(7) Mann, M.; Wilm, M. *Trends Biochem. Sci.* **1995**, *20*, 219−223.
(8) Wilm, M. S.; Mann, M. *Int. J. Mass Spectrom. Ion Processes* **1994**, *136*, 167−180.

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years and has proven to be rugged in a wide variety of practical applications.

At least two other miniaturized electrospray ion sources have been developed. Gale and Smith coupled capillary electrophoresis to electrospray without a liquid sheath flow. In 1992 they described the use of small-diameter capillaries (down to 5 μm inner diameter) terminating in sharp tips etched by hydrogen fluoride.[9] This interface has not been widely adapted in practical applications, however. Emmett and Caprioli integrated a chromatographic packing into the tip of an electrospray needle.[10] Peptides absorbed on this material were step eluted at a flow rate of about 0.3−1.2 μL/min. Sensitivities in the attomole to subattomole range have been reported with that source.

Apart from the different origins of the miniaturized ion sources described by those authors, there are several main differences to the source described here. The nanoES source employs pulled capillaries with a very small spraying orifice and a very low flow rate which in turn leads to very small droplet sizes. It operates at the flow dictated by the electrospray process itself; that is, the flow is not forced by solvent pumps. The nanoES is stable at a flow rate that seems to be lower than that possible or practical in a pumped system, probably because of the very small diameter of the spraying capillary. Rather than loading the sample into an injector loop, the typical mode of operation of the nanoES is to load a 0.2−2 μL volume of analyte solution—often a complex mixture—directly into the needle. As developed here, the nanoES device shares some disadvantages with "off-line" systems but also turns out to have unforseen analytical advantages. Such advantages are the complete absence of any cross contamination, few opportunities for sample loss or system failure, and the ability to choose and change the solvent at will, even during an analysis. Most importantly, and in contrast to other miniaturized electrospray systems, there is virtually unlimited time for tandem mass spectrometric investigations.

We originally chose the name microelectrospray for the device described here.[11] Unfortunately, Emmett and Caprioli had independently named their miniaturized electrospray ion source microelectrospray as well.[12] The miniaturized ion sources are as different from each other as they are to conventional electrospray ion sources and indeed encourage opposite analytical approaches. Therefore, they must be clearly distinguished terminologically and we now call our device nanoelectrospray (nanoES). The name reflects the low nanoliter per minute flow and the droplet size in the nanometer rather than the micrometer range.[13]

Here we give the first practical account of the nanoES source, i.e., the fabrication of the needles, and the operation of the spray. New analytical achievements and strategies made possible by the nanoES source are demonstrated on several examples of practical bioanalytical importance both in protein analysis and in peptide sequencing.

(9) Wahl, J. H.; Goodlett, D. R.; Udseth, H. R.; Smith, R. D. *Anal. Chem.* **1992**, *64*, 3194−3196.

(10) Emmett, M. R.; Caprioli, R. M. *J. Am. Soc. Mass Spectrom.* **1994**, *5*, 605−613.

(11) Wilm, M.; Mann, M. *Proceedings of the 42nd ASMS Conference on Mass Spectrometry and Allied Topics*, Chicago, IL, May 29−June 3, 1994; p 770.

(12) Caprioli, R. M.; Emmett, M. E.; Andren, P. *Proceedings of the 42nd ASMS Conference on Mass Spectrometry and Allied Topics*, Chicago, IL, May 29−June 3, 1994; p 754.

(13) Recently, McLafferty et al. developed a nanoES source with even much decreased flow rate; however, this device keeps the essential design characteristics of the nanoES source and still has to be proven in routine applications.[16]

EXPERIMENTAL SECTION

Chemicals. Horse myoglobin, human carbonic anhydrase, and ovalbumin were purchased from Sigma Chemical Co. (St Louis, MO). The peptide MDMSKDESVDYVPMLD-NH_2 was synthesized in our group on an Applied Biosystems (ABI) 431 A synthesizer (ABD, Foster City, CA) using Fmoc chemistry and purified by reversed phase high-performance liquid chromatography (HPLC). Carbon paste ("Leit-C") to contact the gold-coated glass capillary with the capillary holder was from Neubauer Chemikalien (Münster, Germany). All solvents were HPLC grade. Samples and solutions were not filtered or degassed before use.

Stock solutions of peptide mixtures were prepared by overnight digestion of ~1 nmol of protein with unmodified trypsin (sequencing grade, Boehringer Mannheim) at an enzyme to substrate ratio of 1:20−100 (w/w). Before digestion, proteins were reduced with dithiotreitol (10 mM) in ammonium bicarbonate buffer (0.1 M, pH 8) at 56 °C for 1 h, followed by alkylation with iodoacetamide (55 mM) in the same buffer at room temperature for 30 min in the dark. The resulting digestion mixtures were diluted with 50% methanol to a final concentration of 0.5−1 pmol/μL, and directly transferred into a nanoES spraying capillary.

Mass Spectrometry. All experiments were done on an API III triple−quadrupole instrument (PE-Sciex, Ontario, Canada) with an upgraded collision cell.[14] The pneumatically assisted electrospray source was replaced by the nanoES source constructed in-house. No nebulizer gas or sheath flow was employed. Clean (99.99999%) N_2 gas was used as curtain gas at a flow rate of 0.2−0.6 L/min. Needle voltage was 600−700 V, interface voltage was typically 100 V, and the orifice potential was 55−70 V.

Q_1 scans were performed with 0.1 Da mass step, and the resolution of Q_1 was set so that triply charged ions could be distinguished from doubly charged ones. For operation in the MS/MS mode, Q_1 was set to transmit a mass window of 2 Da and spectra were accumulated with 0.2 Da mass steps. Resolution was set so that fragment masses could be assigned to better than 1 Da and so that doubly charged fragments could be distinguished from singly charged ones. Collision energy was tuned individually for each peptide to obtain the best possible MS/MS spectra.

Manufacture of Capillaries. The essential part of the nanoelectrospray ion source is a gold-coated pulled glass capillary with an orifice of 1−2 μm. Borosilicate glass capillaries from Clark Electromedical Instruments (Pangbourne, GB) of the type GC F120 were used. They were pulled with a microcapillary puller (Model P-87, Sutter Instrument Co., Novato, CA). The pulling was performed in such a way that the capillaries ended in a very fine glass filament. The length of this filament, which had a roughly constant outer diameter of not more than 5−8 μm, was kept under 800 μm to keep its flow resistance small and to prevent its plugging. Once the parameters for the microcapillary puller had been fixed, ~50 capillaries could be pulled within 15 min. A pulling procedure with a two-step cycle was found to result in the most reproducibly shaped glass capillaries.[15] In a first step, the

(14) Thomson, B. A.; Doublas, D. J.; Corr, J. J.; Hager, J. W.; Jolliffe, C. L. *Anal. Chem.* **1995**, *67*, 1696−1704.

(15) Our parameter set for the puller P-87 from Sutter: For the first pulling step, heat 640, pull strength 100, velocity 10, cooling time 250; for the second pulling step, heat 580, pull strength 200, velocity 6, cooling time 200.

1.2 mm o.d. capillaries are pulled to an outer diameter of ~0.5 mm and in a second step to complete separation.

Pulled capillaries were arranged into trays in batches of 25 and the trays mounted into a vapor deposition instrument from Balzers (SCD 020, Balzers, Wiesbaden, Germany). A thin layer of gold was deposited on the full circumference of the needles. The region opposite to the source of gold remained transparent (even though it is covered by a thin film of gold) and allowed viewing the inside of the capillary. The tray was mounted at a distance of 5 cm below a gold target in a small vapor deposition chamber. At a pressure of 0.1 Torr a voltage of 120−140 V was applied causing an electrical current of 15 mA between the gold target and the counterelectrode where the tray was located. Two minutes of sputtering time was sufficient to coat the capillaries with a visible gold layer.

Operation of the Spray. The capillary was mounted in an air-tight stainless steel capillary holder with O-ring fittings to hold the capillary without damaging it. Electrical contact between the metallized capillary and the holder was made by brushing an organic solution of graphite over the junction between needle and holder. The holder was mounted on a small sledge which in turn attached to the arm of the Sciex "articulated ion spray" source. High voltage for spraying was provided from the Sciex voltage supply. The sample was injected directly into the open end of the glass capillaries using long, thin gel-loader tips (Eppendorf, Hamburg). After loading the capillary with 0.2−2 μL of sample solution, the holder was closed and pushed forward to a position 1−2 mm in front of the orifice. The ion source region on the mass spectrometer was viewed by a stereomicroscope at 16-fold magnification (Wild M3B, Leica, Heerbrugg, Switzerland). The source assembly could be manipulated in *x*, *y*, and *z* directions via the micrometer screws of the Sciex ion source.

To prepare for spraying, the needle was briefly touched against the interface plate with all potentials at ground and then centered in front of the mass spectrometer's orifice. A static air pressure of ~0.8 bar was applied at the capillary holder via an air-filled syringe which caused a small droplet to appear at the tip of the needle. A potential of 600−700 V at the needle started the spray. The air pressure was then reduced to avoid forcing the flow for the electrospray. The necessary pressure was adjusted according to the mass spectrometric signal so that a constant stable flow was achieved. No attempt was made to achieve a particular flow rate.

It appears that the main function of touching the interface plate was to slightly open the tip of the capillary from just less than 1 μm i.d. to 1−2 μm i.d. Figure 1 shows a capillary directly after pulling and a used capillary. (Recently, McLafferty et al. found that such capillaries may also be opened by etching them in hydrogen fluoride.[16]) A new capillary was used for each experiment.

Concentration/Desalting into NanoES Needles. Approximately 100 nL of POROS R2 sorbent (Perseptive Biosystems, Framingham, MA) was placed in the tip of a pulled GC 100F-10 (CEI, Pangbourne, GB) capillary. The chromatographic material was not packed and no frit or other microLC assembling was necessary. A new capillary and a new portion of resin were used for each analysis.

(16) Guan, Z.; Aaserud, D.; Valaskovic, G.; Kelleher, N.; McLafferty, F. *Proceedings of the 43rd ASMS Conference on Mass Spectrometry and Allied Topics*, Atlanta, GA, 1995; p 650.

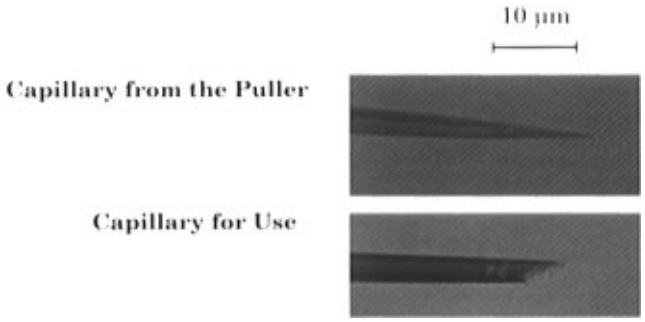


Figure 1. Glass capillaries for the nanoES source (a, top) as made by the microcapillary puller and (b, bottom) after the tip had been touched against the interface plate. Pictures of the glass capillaries were taken with 1000-fold magnification with an oil immersion microscope. The capillary from the puller initially has an orifice much smaller than 1 μm in diameter. After touching the interface plate, the tip is large enough to be used as an emitter for the nanoES source. Note that part of the gold coating has disappeared but that the needle is not chipped. Microscopic pictures of the glass needles were taken with the oil immersion objectives on a Zeiss microscope (Axioplan-Zeiss).

The sorbent was preequilibrated in the sample buffer. Sample solution was loaded onto the capillary using a gel loader pipet followed by gentle centrifugation in a table centrifuge or alternatively by placing the needle in the spraying holder and applying ~1 bar of static air pressure. The sample adsorbed to the POROS material was then washed extensively by passing several microliters of solution (5% formic acid and 5% MeOH in water) through the needle. The capillary was subsequently aligned with its tip into the open end of a spraying capillary and the whole assembly put into a table centrifuge. The sample was step eluted into the spraying capillary with a volume of ~1 μL of solvent (e.g., 60% methanol, 5% formic acid, and 35% water in the case of peptide mixtures).

Unplugging of Needles. Capillaries could be reopened after occasional plugging of the needle when contaminated samples were analyzed. They were moved against the interface plate keeping the interface plate at the same potential as the needle or, if this did not open the needle, with the needle at 500 V and the interface plate at 100 V. So far, it has been possible to reopen all needles by a combination of mechanical and electrical stress.

RESULTS AND DISCUSSION

Ionization and Transfer Efficiency. The overall efficiency in electrospray mass spectrometry can be defined as the number of analyte ions recorded at the detector divided by the number of analyte molecules sprayed. It is thus a combination of desolvation, ionization, and transfer efficiencies into the vacuum system. The nanoES source improves all three factors in comparison to conventional sources.

To determine the overall efficiency, a synthetic peptide was subjected to nanoES and the resulting signal monitored as a function of time. It had previously been found that ~1 in 1300 molecules could be detected.[8] However, in that experiment, isotopic resolution was maintained and no attempt was made at transmitting the maximum number of ions through the quadrupole. In the current experiment, the quadrupole Q_3 was set to rf-only mode and Q_1 to low resolution (peak half-width of 2.5 Da). A 0.5 μL sample of a 0.2 pmol/μL solution of the synthetic peptide MDMSKDESVDYVPMLD was infused. The solution sprayed for

23 min (i.e., a flow rate of 22 nL/min) and an average background-subtracted signal of 73 000 counts/s on the doubly charged peak and 36 000 counts/s on the triply charged peak was recorded. Dividing by the flux of peptide molecules into the source results in an overall transfer of analyte molecules from solution to detector of 1 in 390. When the same experiment is done with the conventional source on our instrument, 1 molecule out of 200 800 sprayed is detected. The overall efficiency is 510 times higher with the nanoES source. The value 1 in 390 is difficult to compare with values in the literature because usually only the transmission for charges rather than for analyte molecules is measured. For a similar mass spectrometer, Smith et. al. reported a value of 1 charge detected for 100 000 charges leaving the needle,[6] and Covey (personal communication) reported a value of 1 in 20 000. These values do not include the ionization efficiency of the electrospray process. If they are taken as upper limits to the overall efficiency as defined above, there is ~2 orders of magnitude difference to the nanoES source. This efficiency gain mirrors the almost 2 orders of magnitude lower flow rate of the nanoES source over conventional sources combined with the generally observed 2−3 times higher ion current at a given analyte concentration.

Several factors may contribute to the high efficiency of converting analyte molecules into analyte ion current in the mass spectrometer. The analyte molecules are separated into different droplets (one molecule per droplet on average in this experiment), which prevents their clustering. The desolvation efficiency could be increased because the droplets are small and monodisperse. The ionization efficiency may be large because the overall charge-to-volume ratio is much higher than for conventional electrospray sources. (The electrical current is ~0.25 μA as compared to ~1 μA in conventional sources whereas the liquid flow is decreased ~2 orders of magnitude.)

In the nanoES ion source, the geometrical setup is favorable for the transfer of a large percentage of generated ions. Since all droplets have sizes below 200 nm they evaporate rapidly and hence the tip of the spraying capillary can be placed at a distance of 1−2 mm in front of the orifice of the vacuum system and in line with the quadrupole axis. The curtain gas can be reduced by roughly 50% to 0.2−0.6 L/min. Thus, a much larger fraction of the ions generated by nanoES is transferred into the vacuum system than in the case of conventional ion sources, where less than 1% of the current generated at the spraying needle is transmitted through the hole in the middle of the interface plate of the mass spectrometer (T. Covey, personal communication) which is the place where the droplets are emitted when the nanoES source is used.

Observation of the mass spectrometric signal when the nanoES source is moved suggests that some of the remaining loss of ions is due to incomplete transmission of the generated ions through the orifice into the vacuum system. However, taking into account other loss factors such as acceptance into and transmission through the quadrupoles, the source described here seems to be within 1 or 2 orders of magnitude of the theoretical limit of converting analyte ions into usable analyte ion current. (Note however that a higher *detection sensitivity* can be achieved by other electrospray ion sources and mass spectrometers by shortening the time during which the peak is detected or by detecting the generated ions in a very efficient way such as in a trapping instrument.)

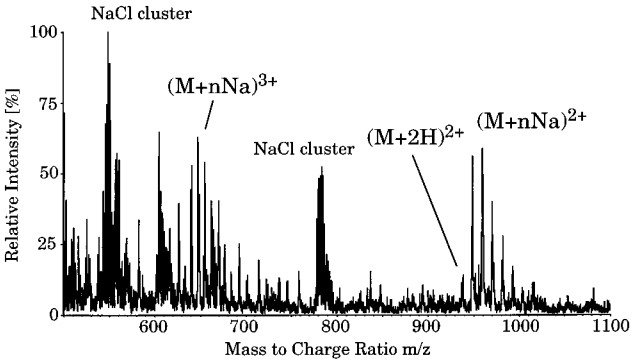
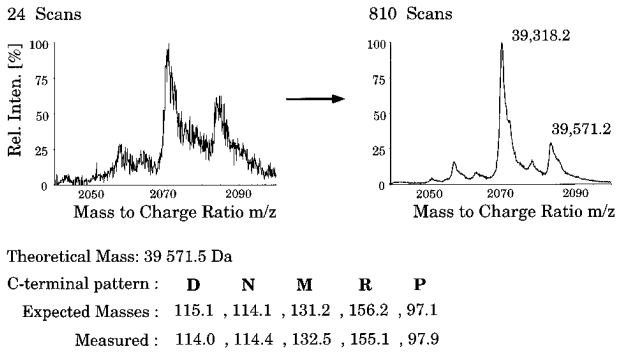


Figure 2. Electrospray spectrum of a 10^{-6} M peptide solution in an aqueous 10^{-1} M NaCl solution. The solution was electrosprayed without any nebulizer or sheath flow assistance. The spectrum is dominated by the salt clusters, but the peptide is still discernable.

Stability of the Spray. The nanoES source disperses the liquid sample purely by electrostatic means, and no assist such as sheath flow or nebulizing gas is used. Nevertheless, it is a very stable source which can spray a wide variety of buffers in positive and negative mode. The stability is illustrated by three experiments: the determination of the stability of a peptide signal during a long time measurement of 20 min, the determination of the stability of the peptide signal when different needles are used, and the reproducibility of the signal intensity between different needles. The standard deviation of the signal intensity of a peptide measured over 20 min with one capillary was 6.1% (sequence MDMSKDESVDYVPMLD-NH_2, concentration 1 pmol/μL in an aqueous methanol solution 1:1). This stability is achieved for all needles. The maximum variation of the signal intensity within one measurement was 8.4% when 15 different needles were subjected to the above experiment. The variation of the signal height between needles was also measured. When the same peptide solution was measured with 25 different needles, the signal intensity had a standard variation of 17%.

Salt-containing aqueous solutions can be sprayed. To demonstrate this point the same synthetic peptide as used for the transmission measurements mentioned above was dissolved in a 100 mM NaCl solution at a concentration of 1 pmol/μL. As can be seen in the resulting mass spectrum in Figure 2, the peptide peak can still be identified. Note that the peptide represents a minor component in the spectrum whose presence is not obvious among the salt clusters. Its signal intensity is only 6.4% of the intensity when measured under identical conditions in a salt-free solution. However, the measurement proves that the spraying and desorption/ionization step in electrospray are not necessarily impossible at very high salt concentrations. Previously such a high tolerance for salt has been the exclusive province of matrix-assisted laser desorption/ionization (MALDI).[17] We have further used the buffer tolerance of the nanoES source to analyze oligonucleotides in the negative mode in aqueous solution at very high pH.[18,19] While such measurements can be possible with conventional sources using, for example, sheath flow or pneumatic assistance, they are—in our experience—easier to perform and control in the current device.

(17) Hillenkamp, F.; Karas, M.; Beavis, R. C.; Chait, B. T. *Anal. Chem.* **1991**, *63*, 1193A−1202A.
(18) Wilm, M.; Talbo, G.; Magris, L.; Mann, M. *Proceedings of the 13th International Mass Spectrometry Conference*; Budapest, 1994; p 68.
(19) Acedo, M.; Tarrason, G.; Piulats, J.; Mann, M.; Wilm, M.; Eritja, R. *Bioorg. Med. Chem. Lett.* **1995**, *5*, 1577−1580.



Theoretical Mass: 39 571.5 Da

C-terminal pattern :	D	N	M	R	P
Expected Masses :	115.1	114.1	131.2	156.2	97.1
Measured :	114.0	114.4	132.5	155.1	97.9

Figure 3. Confirmation of the C-terminal amino acids of a derivative of tissue plasminogen activator. After digestion with carboxypeptidase P, 1 μL of the product mixture was injected into the nanoES source. The spectra were acquired with a step width of 0.1 Da. For precise measurement of the protein masses, the scans were limited to the *m/z* interval of 2020−2130, that is, the $(M + 19H)^{19+}$ ions of the protein mixture. As can be seen on the left, after acquisition of 24 scans the statistics of the ion signals was still so poor that no clear mass assignment could be made. After 810 scans (right) had been added, the mass of the digestion products could be correctly assigned to within 1.5 Da, yielding the C-terminal sequence pattern (ND)(ND)-MRP.

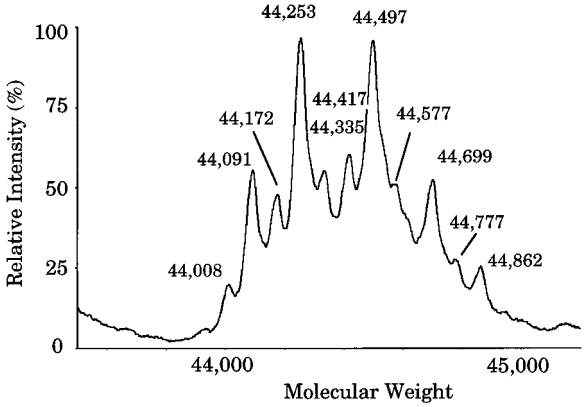


Figure 4. Ovalbumin analyzed with the NanoElectrospray source. A 1 μL sample of an aqueous 5 pmol/μL ovalbumin solution was injected into the nanoES capillary. A total of 19 scans were acquired for this spectrum and the deconvoluted spectrum[31] is shown. The resolution of the individual glycosylated forms was 1300 (fwhm), the same as it was for peptides analyzed under identical mass spectrometric conditions.

Table 1. Comparison of the Measured Ovalbumin Masses to the Calculated Masses of Different Glycosylation States As Indicated[a]

mass		
expected[b,c]	found[b]	oligosaccharide
44 009	44 008	$(Hex)_2(HexNAc)_4$
44 089	44 091	$(Hex)_5(HexNAc)_2$
44 171	44 172	$(Hex)_3(HexNAc)_4$
44 251	44 253	$(Hex)_6(HexNAc)_2$
44 333	44 335	$(Hex)_4(HexNAc)_4$
44 416	44 417	$(Hex)_2(HexNAc)_6$
44 496	44 497	$(Hex)_5(HexNAc)_4$
44 578	44 577	$(Hex)_3(HexNAc)_6$
44 699	44 699	$(Hex)_5(HexNAc)_5$
44 779	44 777	$(Hex)_8(HexNAc)_3$
44 861	44 862	$(Hex)_6(HexNAc)_5$

[a] For an overview of the ovalbumin oligosaccharides see ref 32. [b] Masses are average and expressed in daltons. [c] Mass of ovalbumin polypeptide backbone is 47 250 Da, assuming known posttranslational modification (N-terminal acetylation, one phosphorylation), 42 872 Da.

Advantages for Protein Analysis. The stability of the nanoES source helps in measuring protein masses accurately when protein mixtures and/or low protein concentrations are involved. This is shown here by the enzymatic C-terminal amino acid sequencing of a 39 kDa protein which was needed in the process of obtaining regulatory approval for the protein. The protein was digested by carboxypeptidase P for 1 h in an ammonium bicarbonate buffer, and the resulting protein mixture which contained components at a concentration of ~2 pmol/μL was loaded directly into a nanoES needle. A few initial scans of the mass spectrometer resulted in peaks with very poor signal-to-noise ratios (Figure 3). However, when 810 spectra were added, the peak shape improved to such a degree that the mass differences between the digestion products could be assigned to within 1.5 Da. The resulting sequence pattern was (ND)(ND)-MRP, where amino acids in parentheses are both candidates for that position. A search of a comprehensive database containing more than 160 000 sequences by this pattern revealed that this C-terminal sequence pattern was unique to tissue plasminogen activator.[20] Together with the fact that the initial mass measurement of the protein had shown a single species, this finding established the correctness of the C-terminus of the protein. The experiment was performed on ~100 times less material than needed for C-terminal sequencing by automated chemical methods.[21] To our knowledge, proteins of this size (40 kDa) have not previously been C-terminally sequenced by mass spectrometry.

The high desolvation efficiency achievable with the nanoES source was found to be beneficial when highly glycosylated proteins were investigated. Ovalbumin was chosen as a model glycoprotein with a heterogeneous glycosylation pattern. The glyco groups have high affinity for water molecules, which makes it difficult to measure ovalbumin in a completely desolvated state. The "spectral congestion" caused by the multiple charging series of this 45 kDa protein when overlapped by the heterogeneity and residual solvation had made direct assignment of the glycosylation state virtually impossible in a previous investigation with the same type of mass spectrometer.[22] When analyzed with the nanoES source, the mass resolution of the peaks corresponding to the different glycosylation states was not limited by residual solvation but was the mass spectrometric resolution chosen for the experiment (i.e., the same as for peptides analyzed under the same conditions) (Figure 4). As shown in Table 1, the measured masses could be associated with specific glycosylation states.

Sequencing of Peptides in Unseparated Mixtures. For ESMS analysis, peptide mixtures are usually first separated by reversed phase HPLC and are then on-line detected. This necessity of peptide separation results from the complexity of peptide mixture spectra when convoluted with the charge state distributions of the peptides. Therefore, for the rapid analysis of

(20) The sequence database "nrdb" prepared daily by the group of Sander, EMBL was scanned with PeptideSearch version 2.7 for proteins ending in the C-terminal pattern (ND)(ND)MRP. All 15 matches were tissue plasminogen factor.

(21) See, for example: Miller, C. G.; Hawke, D. H.; Tso, J.; Early, S. In *Techniques in Protein Chemistry VI*; Crabb, J. W., Ed.; Academic Press: New York, 1995; pp 219−227.

(22) Duffin, K. L.; Welply, J. K.; Huang, E.; Henion, J. D. *Anal. Chem.* **1992**, *64*, 1440−1448.

unseparated mixtures, ESMS was not considered a method of choice. However, with the nanoES source, more than 30 min of measurement time is available on 1 μL of solvent, which opens up the possibility of determining the identity of most of the peaks in a complex peptide mass map by fragmenting them.

To demonstrate the practicality of this concept, 2 pmol of a digest of carbonic anhydrase was loaded into the spraying capillary and the peaks in the resulting peptide map were fragmented in turn. Figure 5A shows the peptide map and Figure 5B a typical tandem mass spectrum (the one identifiying the acetylated N-terminus of the protein). A large part of the sequence could be verified by comparing the expected ion series (A, B, Y″ ions and internal series; for the nomenclature see ref 23). After performing the experiment, it was noted that a large peptide (>4 kDa) had not been sequenced. A loading of an additional 1 μL of the peptide mixture provided enough time to fragment several expected charge states of this peptide (Figure 5C). As noted in the figure and in Table 2, almost the complete sequence of carbonic anhydrase could be verified in this experiment.

We have also observed and taken advantage of a unique feature made possible by the current analytical scheme. Large peptides can often be dissociated because they are multiply charged. However, the collision energy must be carefully tuned to obtain spectra with optimal sequence information. This can be done via a collision energy scan while the intensity of a particular fragment ion is monitored. This collision energy optimization was performed for all of the larger peptides (>2 kDa) in the digest. If desired, two or more spectra can be taken of the same peptide to obtain optimal spectra for the high-mass range and for the low-mass range.

Table 2 shows the results of the investigation on carbonic anhydrase; 93% of the sequence could be verified on less than 3 pmol of material and without the use of HPLC. The sequence coverage is as good or better than that of a single LC/MS run. Note that the information provided goes much beyond the information in an LC/MS peptide map, however. In the current experiment, each peptide in Table 2 is identified through its mass and sequence (i.e., in this case comparison of the expected fragmentation series with the actually observed ones). An LC/MS peptide map very often contain ambiguities when, for instance, two tryptic peptides have the same nominal mass and when a mass does not fit the sequence. A complete LC/MS/MS investigation, however, often involves a prohibitive amount of work or sample. Thus, it appears that the concept introduced here could be a useful addition or alternative to LC/MS or LC/MS/MS methods. In general, the peptide map of a protein under investigation may not yield as good a coverage as in the case of the model protein presented here. However, the investigation of a complementary enzymatic digest with the method described here would be a rapid way to cover a large part of the sequence.

It would be a major limitation of the strategy of direct peptide mixture analysis if only sample solutions pure enough to be electrosprayed could be analyzed. To be able to analyze contaminated samples and to concentrate digestion mixtures into a 1 μL volume, we have developed a rapid desalting/concentration technique (see Experimental Section). This technique is applied in a final example to the identification of protein from a one-dimensional gel. This protein had been found and cloned by

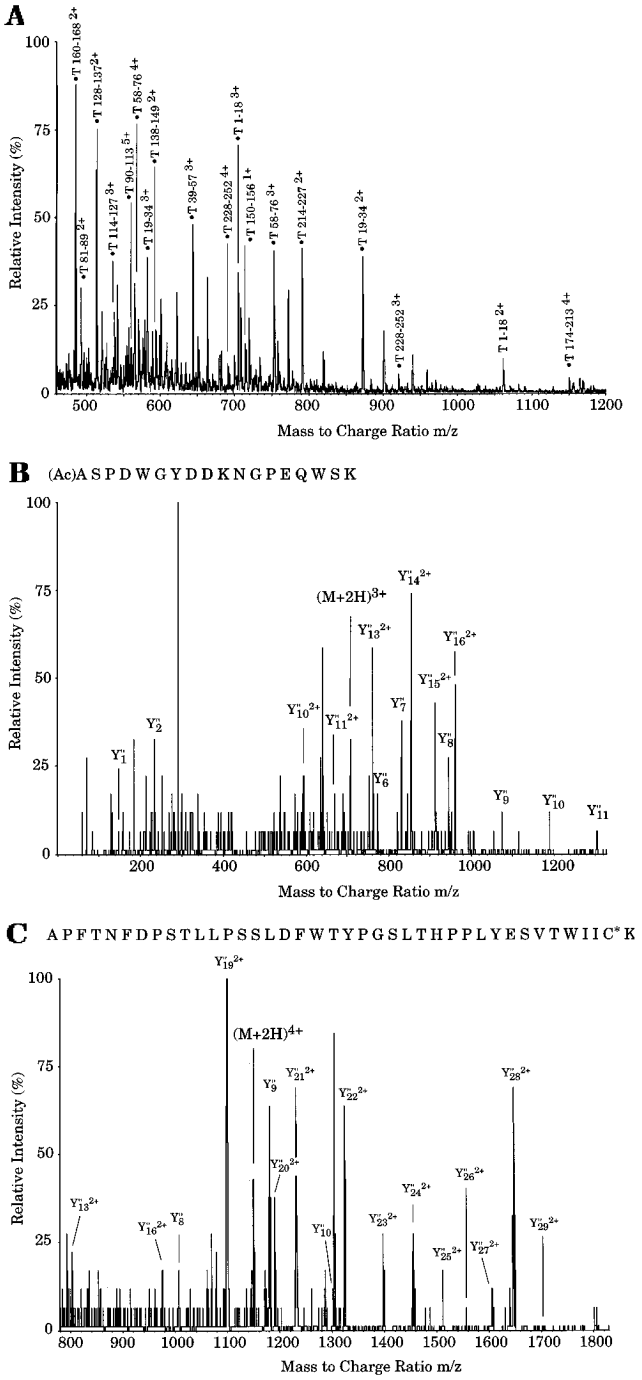


Figure 5. nanoES analysis of a tryptic digest of human carbonic anhydrase (SWISSPROT accession number P00915). A 2 μL sample of a 1 pmol/μL solution of the unseparated peptide mixture was loaded into the electrospray source and investigated by mass spectrometry and tandem mass spectrometry for 80 min. Peaks are labeled by the amino acid residues according to the sequence (see also Table 2). Labels preceded by "•" were sequenced by tandem mass spectrometry. Panel B shows the tandem mass spectrum of the peak at *m/z* 708.3, the triply charged N-terminal peptide. The Y″ ion series are marked in the spectrum and suffice to clearly identify the peptide. Panel C shows the fragmentation of the largest tryptic peptide. The quadruply charged ion at *m/z* 1150 was dissociated. As in (C), a series of Y″ ions clearly identifies the peptide, and further ion series are not labeled in the spectrum.

genetic means as a factor involved in nuclear transport,[24] and it was to be isolated and identified for biochemical studies. To this

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Table 2. Peptides Sequenced from Human Carbonic Anhydrase (See Figure 5)

mass		charge	
expected[a]	found[a]	state fragmt	sequence
969.6	969.6	2+	VLDALQAIK
984.4	984.4	2+	GGPFSDSYR
1611.8	1611.9	3+	YSAELHVAHWNSAK
1741.9	1743.0	3+	LYPIANGNNQSPVDIK
1185.7	1185.6	2+	ADGLAVIGVLMK
2474.2	2475.6	4+	TSETKHDTSLKPISVSYNPATAK
1928.0	1928.4	3+	HDTSLKPISVSYNPATAK
2758.4	2759.4	4+	SLLSNVEGDNAVPMQHNNRPTQPLK
2120.9	2121.3	3+	AcASPDWGYDDKNGPEQWSK[b]
713.4	713.3	1+	VGEANPK
2255.0	2255.4	3+	EIINVGHSFHVNFEDNDNR
1579.8	1579.6	2+	ESISVSSEQLAQFR
1741.9	1742.8	2+	LYPIANGNNQSPVDIK
2758.4	2759.1	3+	SLLSNVEGDNAVPMQHNNRPTQPLK
1025.5	1025.5	2+	YSSLAEAASK
4597.2	4598.8	4+	APFTNFDPSTLLPSSLDFWTYPGSLTHPPLYESVTWIIC*K[b]
2795.3	2796.5	5+	LFQFHFHWGSTNEHGSEHTVDGVK

[a] Masses are neutral and monoisotopic and expressed in daltons. [b] Ac is the acetyl group, C* is *S*-acetamidomethyl-cysteine; underlined amino acids were found as continuous Y″ or B ion series. (Note, however, that the rest of the peptide sequence is verified implicitly by its mass.)

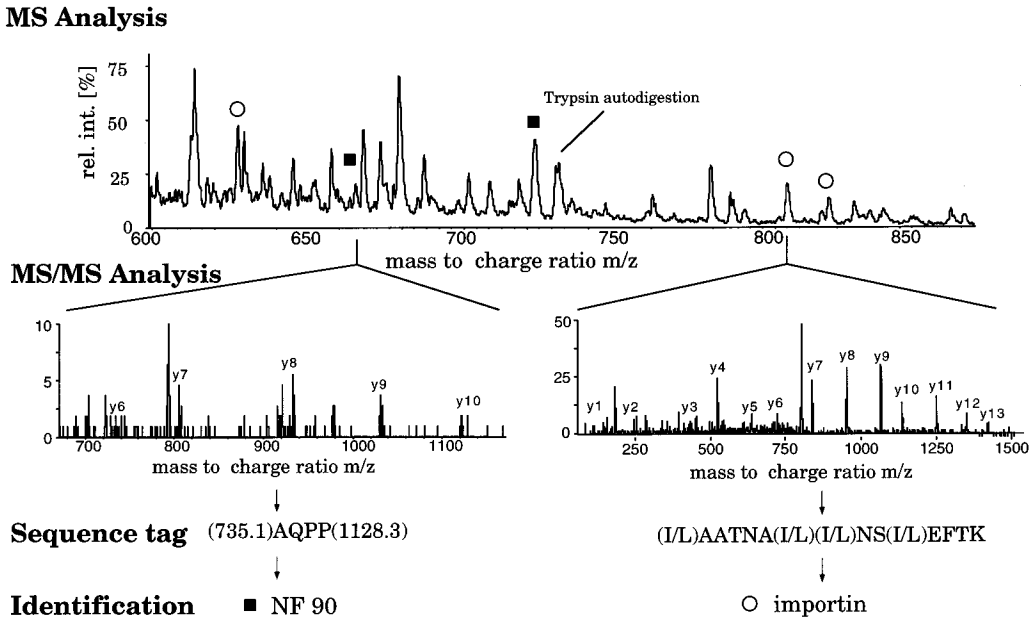

Figure 6. Peptide map obtained after in-gel digestion of a protein with a role in nuclear transport. The top portion shows a part of the peptide mass map in which five peptides had been subjected to tandem mass spectrometry. The thee peaks marked by open circles originate from "importin" the protein to be verified. The three peaks labeled by black boxes identified "NF-90" (Genpept accession number U10324), another 90 kDa human protein. A total amount of ~4 pmol of protein (for both proteins together) was present on the gel as judged by the intensity of Coomassie staining.

end, proteins were affinity purified and the resulting protein fraction, which presumably contained the protein of interest, was separated on a one dimensional polyacrylamide gel. After the protein had been digested in gel[25] and the peptides had been extracted, the resulting 10 μL of peptide mixture was adsorbed on a small volume of POROS material in a pulled capillary needle. There the peptides were washed extensively and step eluted into the spraying capillary. The peptides in the resulting map were sequenced, (Figure 6) and the putative protein was identified in a database search.[26] Surprisingly, several other peaks mapped to an unsuspected protein which was present in what seems to be a 1:3 ratio with the major protein. The problem-free analysis of peptide material extracted from a polyacrylamide gel without HPLC purification and the unique identification of two proteins from the unseparated peptide maps demonstrates the power of the approach which we suggest here.

CONCLUSION AND PERSPECTIVE

The nanoES ion source has been shown to possess a number of unique analytical features. These include freedom to choose the solvent in a wide range of composition and pH and an unprecedented tolerance to salt contamination. Advantages of nanoES in protein analysis include the possibility of long signal averaging to obtain accurate mass measurements in protein

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mixtures and the possibility to desolvate, for example, glycoproteins better. The analysis of unseparated peptide mixtures shows the possibility for rapid and sensitive analytical strategies.

The tolerance toward buffer compositions is an important characteristic of the ion source for the analysis of noncovalent interactions. For this kind of experiment, the buffer composition is dictated by the need to keep the noncovalent interactions intact in solution. Analysis of buffered solutions is a strength of the nanoES source. Furthermore, as Smith and Light-Wahl pointed out,[27] the analysis of noncovalent interactions necessitates careful optimization of interface conditions which in turn takes much measurement time. Thus the nanoES source may be ideal for this kind of investigation.[28] From a mechanistic standpoint, it is interesting to speculate on whether there will be advantages in the fact that only a few proteins and ligand pairs are present in each initial droplet.

The nanoES device has now been supplied to a number of laboratories and it has been interfaced to trapping instruments[29,30] and time-of-flight instruments and is being coupled to magnetic sector instruments. Derivatives of the source are being developed. In the near future we hope to use the strength of the nanoES source in the high-accuracy sequencing of long (>15 amino acid sequences.

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