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Separation and Identification of Peptides from Gel-Isolated Membrane Proteins Using a Microfabricated Device for Combined Capillary Electrophoresis/Nanoelectrospray Mass Spectrometry

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The coupling of microfabricated devices to nanoelectrospray mass spectrometers using both a triple quadrupole and a quadrupole time-of-flight mass spectrometer (QqTOF MS) is presented for the analysis of trace-level membrane proteins. Short disposable nanoelectrospray emitters were directly coupled to the chip device via a low dead volume connection. The analytical performance of this integrated device in terms of sensitivity and reproducibility was evaluated for standard peptide mixtures. A concentration detection limit ranging from 3.2 to 43.5 nM for different peptides was achieved in selected ion monitoring, thus representing a 10-fold improvement in sensitivity compared to that of microelectrospray using the same chip/mass spectrometer. Replicate injections indicated that reproducibility of migration time was typically less than 3.1% RSD whereas RSD values of 6−13% were observed on peak areas. Although complete resolution of individual components is not typically achieved for complex digests, the present chip capillary electrophoresis (chip-CE) device enabled proper sample cleanup and partial separation of multicomponent samples prior to mass spectral identification. Analyses of protein digests were typically achieved in less than 1.5 min with peak widths of 1.8−2.5 s (half-height definition) as indicated from individual reconstructed ion electropherograms. The application of this chip-CE/QqTOF MS system is further demonstrated for the identification of membrane proteins which form a subset of the *Haemophilus influenzae* proteome. Bands first separated by 1D-gel electrophoresis were excised and digested, and extracted tryptic peptides were loaded on the chip without any further sample cleanup or on-line adsorption preconcentration. Accurate molecular mass determination (<5 ppm) in peptide-mapping experiments was obtained by introducing an internal standard via a postseparation channel. The analytical potential of this integrated device for the identification of trace-level proteins from different strains of *H. influenzae* is demonstrated using both peptide mass-fingerprint database searching and on-line tandem mass spectrometry.

The emergence of proteomic sequencing projects poses significant demands for novel analytical techniques which can provide rapid and sensitive identification of proteins from subpicomole amounts of proteolytic digests.[1] Both nanoelectrospray and matrix-assisted laser desorption ionization (MALDI) mass spectrometry have been used to rapidly identify gel-isolated proteins on the basis of their characteristic mass spectral signatures and correlation with structural information obtainable from protein databases.[2−6] The preferred approach to trace-level peptide sequencing uses nanoelectrospray and tandem mass spectrometry (MS-MS) to yield sequence segments, which, in combination with the mass of the precursor ion, provides low probability of false positive identification. Different approaches were developed to facilitate the interpretation of MS-MS data and correlation with protein databases.[7−9] Alternative approaches to peptide identification use partial amino acid sequences plus the masses of the precursor ion and of the unidentified N- and C-terminal segments.[10−12]

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Although protein identification can be achieved by direct nanoelectrospray, the mass spectral sensitivity can be adversely affected by the presence of salts used in proteolytic digestion or by the simultaneous ionization of a large number of different peptides isolated from these digests.[11] Reliable detection of proteolytic fragments by nanoelectrospray is contingent upon the successful cleanup of the gel-separated band to purify subpicomole amounts of peptides. Such desalting and sample cleanup procedures often require the use of small-scale adsorptive media to remove buffers and gel contaminants that can suppress the analyte ionization. These procedures are often time consuming and can result in the loss of hydrophilic peptides (e.g., phosphopeptides). The combination of a rapid on-line separation method with mass spectrometry can thus offer a unique advantage in situations where both sensitivity and selectivity are desired.

Significant advances in automation will be needed to supply the throughput requirements of applications involving the rapid identification of proteins. Microfluidic devices[13−16] may be able to meet these needs in the future, as the individual components presently fabricated on chips for performing capillary gel electrophoresis, electrochomatography, cell lysis, or biochemical reactions become integrated together. Such microchip systems may then be coupled to mass spectrometers via electrospray to automate processing and introduction of biological samples into the mass spectrometer for rapid protein identification through the use of database-searching routines.[17−21] At present, the performance of microchip electrospray interfaces still requires improvement, to provide the necessary limits of detection (LOD) and separation power. Additionally, the combination of this potentially rapid, high-resolution separation method with a scanning quadrupole mass spectrometer often requires that some compromises be made in order to attain adequate speed and detection limits. Recent developments in quadrupole/time-of-flight instrumentation (QqTOF) have enabled the rapid acquisition of wide mass ranges (>10 scans/s) with mass spectral resolution exceeding 5000. Hsieh has demonstrated the value of these instruments in protein analysis by coupling capillary electrophoresis (CE) to a TOF instrument for the rapid separation of peptide standards.[22] The speed of these instruments is well suited to the separation speed seen with microchip CE, so that an exploration of the coupling of a QqTOF mass spectrometer to a chip-based electrospray device is required. Given the present stage of microfluidic device performance, the use of a chip may be considered to aid in sample cleanup of a protein or a peptide digest, through a partial separation of the components and isolation of the peptides from the digestion reagents and buffers before their introduction into the QqTOF mass spectrometer.

Our preliminary studies with a microfluidic device, comprising a low-dead-volume connection to an electrospray capillary for performing nanospray (spray at nanoliter flow rates), gave lower separation efficiencies and poorer detection limits (typically 0.2−2 μM) than conventional nanospray CE.[23,24] In this report, we present methods to improve this performance considerably through the use of surface coatings and a gold-coated nanoelectrospray tip, so that the microchip performance becomes similar to that of conventional nanoelectrospray CE in terms of LOD and resolution per meter yet more rapid due to the greater ease of creating short capillary lengths. The coupling of chip-CE to nanoelectrospray mass spectrometry (chip-CE-nESMS) using a QqTOF instrument is demonstrated in the present report. The application of such a device enabled the identification of peptides in tryptic digests of glycoproteins and further demonstrated that chip-based electrospray devices can be used with the QqTOF design.

A step was also taken toward the use of microchip-based electrospray devices in the development of more rapid methods for the study of the proteome. Extracts of membrane-bound proteins from *Haemophilus influenzae* representing a subset of its total proteome, and identification of these components can provide valuable information in the design of vaccines against this bacterial pathogen. Specific extraction protocols were developed to enrich protein extracts for these hydrophobic cellular components. This was followed by a one-dimensional SDS-PAGE separation of the proteins, tryptic digestion of excised spots, and introduction into the microfluidic device for the final sample cleanup prior to electrospraying into the QqTOF mass spectrometer. Both peptide mass fingerprinting and sequence tag approaches were used for the identification of surface-associated peptides observed in the genomic Rd and pathogenic Eagan strain of *H. influenzae*.

EXPERIMENTAL SECTION

Chemicals and Materials. A fused-silica capillary was purchased from Polymicro Technologies (Phoenix, AZ) and Teflon tubing from LC Packing (San Francisco, CA). Peptide standards were purchased from Sigma Chemical Co. (St. Louis, MO) and used without further purification. Gold-electroplating solution was prepared with 24K Bright English gold plating salts (Grobet File Co. of America, Inc., Caristadt, NJ). [(Acryloylamino)propyl]-trimethylammonium chloride (BCQ) was obtained from Chemische Fabrik Stockhausen (Krefeld, Germany). 7-Oct-1-enyltrimethoxysilane was purchased from United Chemical Technologies Inc. (Bristol, PA). *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED) was obtained from Aldrich Chemical Co. Inc. (Milwaukee, WI) and formic acid from BDH Inc. (Toronto, ON, Canada).

Device Fabrication, BCQ Coating, and Nanoelectrosprayer Construction. The microfluidic devices were fabricated at the Alberta Microelectronic Centre (Edmonton, Alberta, Canada), as

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described previously.[23,25] Both the chips and the nanoelectrospray emitters were covalently modified with BCQ coating to prevent analyte adsorption on the silica walls.[26] To insert the emitter into the microfabricated devices, a flat-bottomed hole (200 μm i.d.) was drilled at the exit of separation channel so that the end of the capillary could be butted to the device with minimal dead volume.[22] The exit end of the nanoelectrospray emitter (185 μm o.d. × 50 μm i.d.) was tapered to 60 μm o.d. (ca. 15 μm i.d.) using a laser puller (Sutter Instruments, Novato, AZ) and cut to approximately 3 cm length. The tapered ends of the emitters were first coated with an Inconel alloy using an Edwards 19e thermal evaporation system (Edwards, Wilmington, MA). A layer of gold (typically 1 μm thickness) was subsequently deposited on the metallized emitters at a reduced pressure of 9.6×10^{-6} mbar using the Edwards 19e high-vacuum coater. The sputtered gold coating was supplemented by electroplating.[27] The gold-coated portion of the tip was covered with silver conductive adhesive to within 1 cm of the tapered tip to prevent removal of the coating during the electroplating process. The gold-plating solution (0.5% gold plating salts in deionized water) was gently stirred at room temperature. A 1 cm section of the capillary was immersed in the plating solution, and a constant flow (0.5 μL/min) of filtered deionized water was maintained to prevent blockage. The plating current for a single tip was maintained at 0.2 mA for 45−60 min. The electroplating procedure removed major surface imperfections and produced a capillary with a smooth reflective gold coating. The tips coated with this procedure were used for at least 3 days without any degradation in performance.

Isolation and Preparation of Membrane Proteins. *H. influenzae* strain Eagan, a serotype b clinical isolate from the National Research Council culture collection (NRCC 4247), and a nonpathogenic (Rd) of the same bacterium were grown separately at 37 °C on chocolate agar plates (Quelab, Montreal, PQ, Canada).[28] The cells were scraped off the plates and lysed by mechanical shearing on an Emulsiflex. A tablet containing a cocktail of protease inhibitors was added to prevent proteolysis. The solution was centrifuged at 3020*g* for 20 min to remove the large particles such as intact cells. The pellet was discarded, and the supernatant was centrifuged at 100000*g* for 1 h. The supernatant was discarded, and the pellet was resuspended in 7 M urea, 2 M thiourea, 4% CHAPS, 2 mM tributylphospholine, 40 mM Tris, and 2% IPG buffer 3-10. All but the most hydrophobic proteins were soluble in this solution. The resoluting solution was centrifuged at 10000*g* for 1 h. The supernate was removed, and the pellet was solubilized by boiling in Laemmli buffer (100 μL) for 5 min. The proteins in the Laemmli buffer were separated by 1D SDS-PAGE (10% T, 20 cm × 20 cm × 1.5 mm). Approximately 2 μg of proteins was applied in each of the most concentrated lanes (1 and 0.5 μg in the other lanes). The proteins were visualized by silver staining as described previously.[11] The protein bands were excised, reduced/alkylated, and in-gel digested with trypsin. The gel pieces were extracted first with 5% acetic acid solution and then with 50% acetonitrile/5% acetic acid solution (100 μL each, with sonication for 10 min). The extracts were combined with the original digest, and the sample was evaporated to dryness on a Savant preconcentrator. The residues were dissolved in 10 μL of running buffer, and approximately 4 μL of this solution was loaded onto the sample reservoir.

Chip CE-nESMS. A 0.1 M formic acid solution was used for the separation of peptide standard mixtures and protein digests with a BCQ-coated chip and nanoelectrospray emitter. All aqueous solutions were filtered through a 0.45 mm filter (Millipore, Bedford, MA) before usage. The chip device was maintained in position by a chip holder as described previously.[24] A schematic representation of this device is shown in Figure 1. Small plastic pipet tubes were inserted through small holes made in the center of septa (Thermogreen LB-1 from Supelco or Septa 77 from Chromatographic Specialties) before assembly and used as sample or buffer reservoirs. Typically, separation (well B) and waste (well C) reservoirs were filled with 30 μL of separation buffer using pipets, whereas 4 μL of the protein digest was placed into the sample reservoir (well A) using a syringe. A Teflon tube (180 μm i.d.) was inserted into the center of septa for well D and used to seal a capillary transfer line. In this configuration, the chip lay on the Teflon support and a Plexiglas top was used to compress the septa to provide an airtight seal between the sample/buffer reservoirs and the chip device. Each well was tightly sealed, thereby preventing any risk of blockage or contamination from epoxy resin. Sample injection was conducted by applying −2.0 kV at well A while well C was grounded. The volume of the sample plug varied depending on the applied voltage and duration of injection since no sample-plug shaping voltage was applied to the separation channel.[16] In the present experiment, the injection volumes were estimated to be 1−3 nL on the basis of the widths and variances of peaks observed in replicate injections of peptide standard solutions.[23]

Mass spectrometric experiments were conducted using a PE/Sciex API 300 triple quadrupole and a prototype QqTOF instrument (Perkin-Elmer/Sciex, Concord, ON, Canada). The interface conditions were optimized by infusion of a 1 μg/mL solution of angiotensin I from the side well D. To facilitate accurate mass measurements during the separation, this peptide solution was used as an internal standard and was introduced at a flow rate of 50 nL/min using a syringe pump (Harvard). It is noteworthy that the flow rate of the separation channel was significantly higher (ca. 200 nL/min) than that of the side channel and that no significant effect on the migration time or separation performance was noted (variation of less than 10%). Under the present configuration, no voltage was applied to channel D and was left floating. Separations were conducted by applying −1.5 kV at well B while the nanoelectrospray emitter was maintained at a voltage of 2.7 kV. A high-tension lead was attached to the nanoelectrospray emitters and maintained in position using a thin coat of silver conductive paint.

Tandem mass spectrometry experiments were performed using the QqTOF hybrid mass spectrometer.[29] Collision-induced dissociation (CID) of selected precursor ions identified from a first sample injection was achieved using argon as the collision gas at

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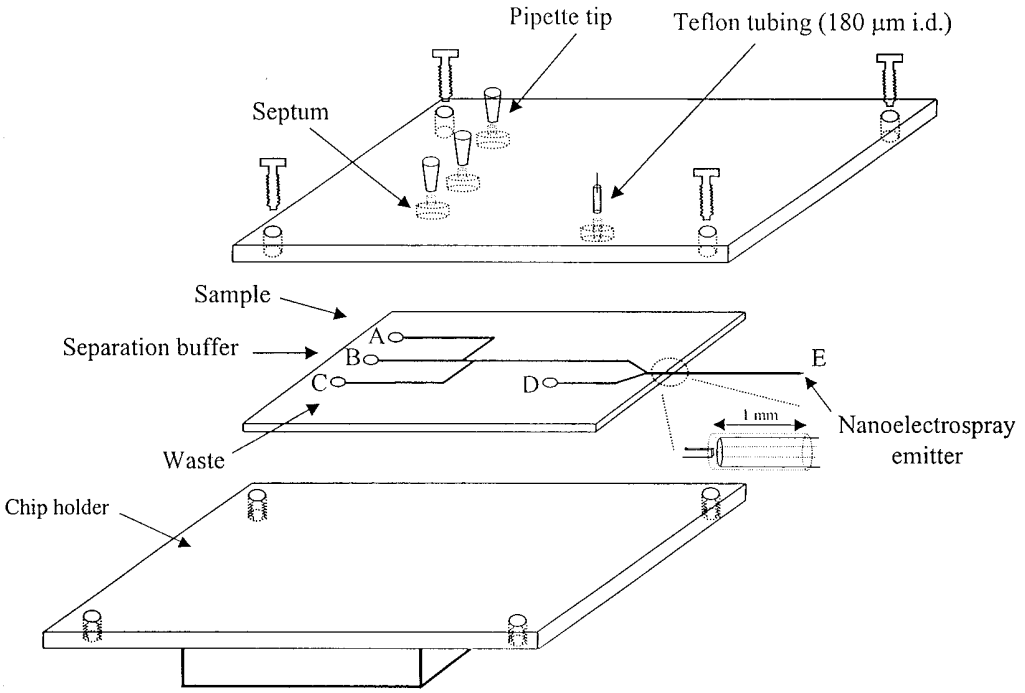

Figure 1. Schematic representation of the chip-CE configuration using a disposable nanoelectrospray emitter (device PCRD2) interfaced to either a triple-quadrupole or a hybrid QqTOF mass spectrometer.

collision energies of typically 40−60 eV (laboratory frame of reference). Fragment ions formed in the rf-only quadrupole were recorded by the TOF mass analyzer.

Database Searching with Mass Spectrometric Data. Accurate peptide masses were determined using internal standardization on the QqTOF instrument and were transferred into the PeptideSearch program.[10] The list of peptide masses was searched against a nonredundant protein sequence database containing over 257 000 entries downloaded from the European Bioinformatic Institute web site (ftp://ftp.ebi.ac.uk/pub/databases/peptidesearch/). Parameters for all searches assumed that masses corresponded to tryptic peptides and that cysteine residues were converted to *S*-(carbamidomethyl)cysteine. All peptide masses were considered monoisotopic, and the maximum deviation between the calculated and measured masses was set to <10 ppm. The search did not impose any restrictions on the species of origin, and the range of protein masses was set to 0−300 kDa. Alternatively, the search was conducted using the peptide sequence tag approach where the precise molecular mass of a given tryptic peptide and the mass of a partial amino acid sequence derived from its MS-MS spectrum were used to retrieve potential protein candidates.

RESULTS AND DISCUSSION

Preliminary investigations using uncoated nanoelectrospray emitters coupled to a microfabricated device via a low-dead-volume connection enabled rapid peptide analyses (<2 min) with high-nanomolar detection limits and moderate separation efficiencies.[23] This relatively lower performance was associated with the selection of electrophoretic conditions facilitating both the separation and the ionization of the analytes when using bare fused-silica surfaces. The use of ammonium acetate enabled the separation of individual analytes as anions though the mass spectrometer was set for positive-ion detection. The peak widths (half-height definition) for individual components typically ranged from 3.5 to 10 s, thus resulting in separation efficiencies of less than 3500 theoretical plates for a 5 cm length channel. Alternate buffer systems containing 0.1 M HCOOH in 20% aqueous methanol generally provided a 10-fold improvement in sensitivity but resulted in poor EOF reproducibility, analyte adsorption, and lower separation efficiencies. Furthermore, the requirement for regular chip reconditioning with caustic solutions further limits the practical application of uncoated devices.

To minimize analyte adsorption on bare silica walls and to eliminate the need for periodic surface reconditioning, we coated both the chip device and the nanoelectrospray emitter with BCQ, a quaternary amine reagent imparting a permanent positive charge on the surface. Since the direction of the EOF is reversed (anodal EOF), separate power supplies were required to effect separation and ionization of the analytes. Gold-coated nanoelectrospray emitters were also required to provide proper application of the ionization voltage.

Analytical Characteristics of the Chip-CE-nESMS Interface Coated with BCQ. To compare the sensitivity of the present interface to that obtained previously using a microelectrospray interface,[23] the BCQ-coated chip devices were first coupled to an API 300 triple-quadrupole mass spectrometer. A separation of a mixture of five peptide standards (10 μg/mL each) is presented in Figure 2. This example was selected for comparison purposes rather than illustrating the limit of detection of the present system. The separation was achieved using an effective field strength of 600 V/cm and an electrolyte composed of 0.1 M HCOOH. A15 s electrokinetic injection at −2 kV resulted in the on-column introduction of 10−30 pg of each peptide. The variability in sample loadings for the different peptides is correlated with their inherent differences in electrophoretic mobilities, μ_{ep}; longer injection times

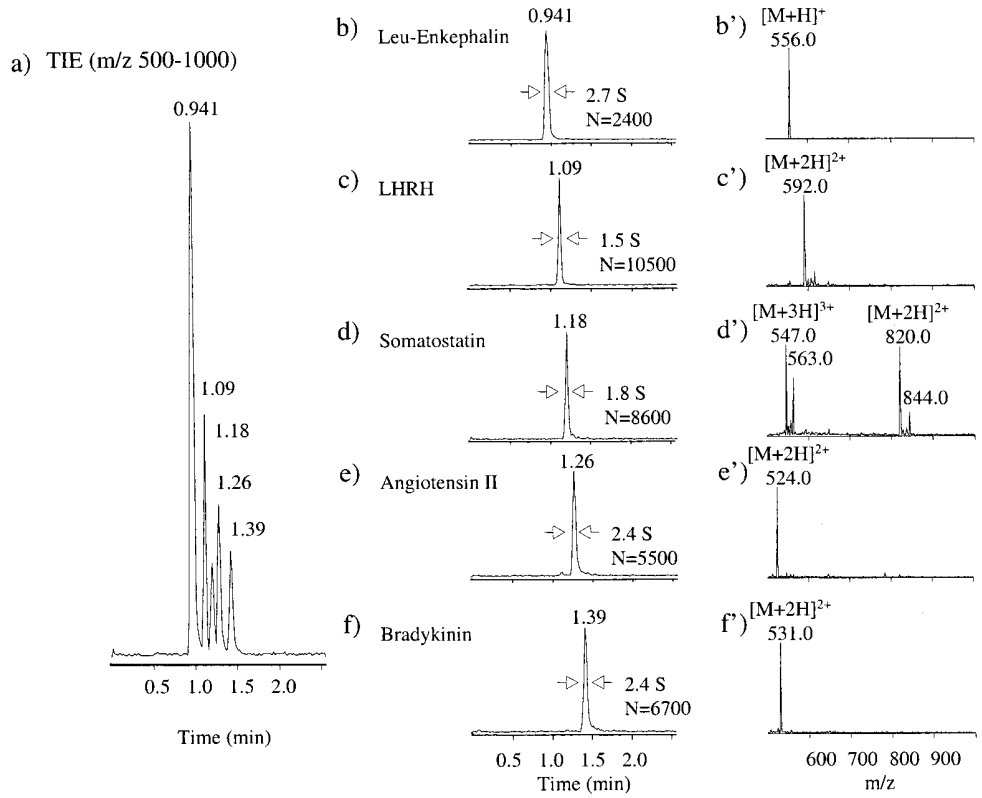

Figure 2. Chip-CE-nESMS analysis of a mixture of synthetic peptides (10 μg/mL each) using a triple-quadrupole instrument. (a) TIE (*m/z* 500−1000); (b−f) RIE for (b) leu-enkephalin (*m/z* 556), (c) LHRH (*m/z* 592), (d) somatostatin (*m/z* 547), (e) angiotensin II (*m/z* 524), and (f) bradykinin (*m/z* 531); (b′−f′): the extracted mass spectra for the corresponding peptides. The microfabricated device was coated using BCQ and connected to a 3 cm BCQ/gold-coated nanoelectrospray emitter (15 μm i.d). Electrolyte: 0.1 M formic acid. A voltage of −1.5 kV was applied to well B (see configuration) for separation, and +2.7 kV was applied at the emitter. The internal standard solution was applied from well D with a flow rate of 50 nL/min.

favor the introduction of analytes of lower μ_{ep} values such as leu-enkephalin (note that the direction of μ_{ep} is opposite to that of the EOF).

The total ion electropherogram, TIE (Figure 2a), shows baseline separation of all peptides in less than 1.4 min analysis time. Individual components present in this mixture were identified from their corresponding reconstructed ion electropherograms (RIE's), as indicated in Figure 2b−e for the singly or doubly protonated molecules of leu-enkephalin (*m/z* 556), LHRH (*m/z* 592), somatostatin (*m/z* 820), angiotensin II (*m/z* 524), and bradykinin (*m/z* 531). The peak widths (half-height definition) for the individual components ranged from 1.5 to 2.7 s, which provided separation efficiencies of 2400−10 500 theoretical plates (2600−9600 plates/min) for a total separation channel length of 7 cm or an equivalent of 78 500−150 000 plates/m. The unusually low separation efficiency observed for leu-enkephalin is related to sample overloading, as the electrophoretic bias favors larger loadings of lower mobility analytes, thus resulting in noticeable peak broadening. The separation performance obtained here is consistent with recent reports describing combined devices using an attached capillary of 15 cm where an average of ∼30 000 theoretical plates was obtained.[24] Devices of shorter separation length (serpentine of 10 cm with a built-in nebulizer) provided lower separation efficiencies, i.e., 70 000 vs 300 000 plates/m for a chip with a transfer line.[30] In a separate paper by Lazar et al.,[31] who described a device more akin to that used in the present study, lower separation efficiencies of ∼2200 plates (44 000 plates/m) for a total channel length of 5 cm (2 s peak widths) were reported.

The extracted mass spectra for each peptide are presented in Figure 2b′−f′ and clearly show the singly and multiply protonated ions of the corresponding peptides. The only exception to this is for somatostatin, for which an extra component of 48 Da higher mass than expected was observed (Figure 2d′). This unexpected peptide was related to the original somatostatin sample and could possibly arise from the hydroxylation of side chain lysine and tryptophane residues rather than an artifact of the ionization conditions.

The sensitivity of the chip-CE-nESMS interface was investigated using serial dilutions ranging from 1 to 1000 ng/mL of each peptide in the selected-ion-monitoring (SIM) mode, and the results obtained are summarized in Table 1. In all cases, good linearity, with a correlation coefficient of $r^2 = 0.971-0.994$ was found over analyte concentrations ranging from 1.0 ng/mL to 1 μg/mL. Low-nanomolar detection limits were achieved for all five peptide

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Table 1. Limits of Detection and Peak Parameters Obtained by Using the SIM Acquisition Mode

peptides	m/z	LOD (nM)	migration time[a]		peak area[a]	
			av (s)	RSD (%)	av ($\times 10^5$)	RSD (%)
leu-enkephalin	556.3	3.20	56.7	2.93	1.34	10.1
LHRH	592.2	28.6	64.0	3.05	0.476	13.2
somatostatin	820.0	43.5	66.9	2.77	0.151	6.10
angiotensin II	524.0	20.3	68.8	3.00	0.868	8.14
bradykinin	530.9	17.6	74.5	1.93	1.57	11.2

[a] Based on replicate of five separate injections.

standards, which corresponds approximately to a 10-fold enhancement of sensitivity compared to that obtained previously using the same instrument.[24] This in turn represents a 2−3-fold improvement in sensitivity over that described earlier for CZE-nanolectrospray mass spectrometry.[26] Replicate injections ($n = 5$) of a 0.1 μg/mL solution of the same peptide mixture described above were analyzed to evaluate the reproducibility of the chip-CE-nESMS system. Table 1 shows the relative standard deviations (RSD's) obtained for migration times and peak areas of the peptides. Good reproducibility of migration times was obtained, with RSD values of 1.9−3.0% for all peptides, whereas RSD values of 6−13% were observed on peak areas. It is noteworthy that peak area measurements deviated more markedly from the mean values following successive injections. This variability in sample loading was associated with the electrokinetic injection and the relatively small sample volume used in the present experiment (30 μL or less). Changes in pH and composition of the sample vial were previously reported when electrokinetic injections were used.[32] The sample loading here is controlled by the duration of the electrokinetic injection rather than the geometrical size of the injector itself.[16]

Chip-CE-nESMS Analyses Using a QqTOF Hybrid Mass Spectrometer. The enhanced separation efficiencies observed in the present chip configuration imposed significant demands on the scanning mass spectrometer in terms of acquisition speed, mass range, and sensitivity. The analytical performance of a quadrupole mass analyzer can be significantly compromised in situations where mass ranges spanning across 1000 m/z units and scanning speeds of less than 1 s/scan are sought. In contrast, the faster duty cycle combined with the inherent mass spectral resolution typically achievable on the TOF instrument provides a significant advantage for rapid analyses of complex protein digests.

The analytical potential of the present chip-CE device coupled to a prototype QqTOF instrument was first investigated for the separation of tryptic peptides from the seed lectin of *Phaseolus vulgaris* L. In this particular application, 100 μg of the lectin was digested overnight with trypsin, the digest was evaporated to dryness, and the residue was dissolved in 1 mL of 0.1 M HCOOH. The TIE of the corresponding analysis is presented in Figure 3a. Although complete resolution of individual tryptic peptides could not be achieved using the present system, a close inspection of peaks eluting between 45 and 65 s enabled the identification of a number of multiply protonated peptides from the mass spectra. The RIE's for selected peptide ions at m/z 876 and 663 are shown in Figure 3a as an example. Consistent with those observed previously for peptide standards (Figure 2), the peak widths of individual RIE's shown in Figure 3a were typically 2 s. Obviously, the unique advantage of the present system relies on the selective mass spectral resolution of partly separated electrophoretic peaks.

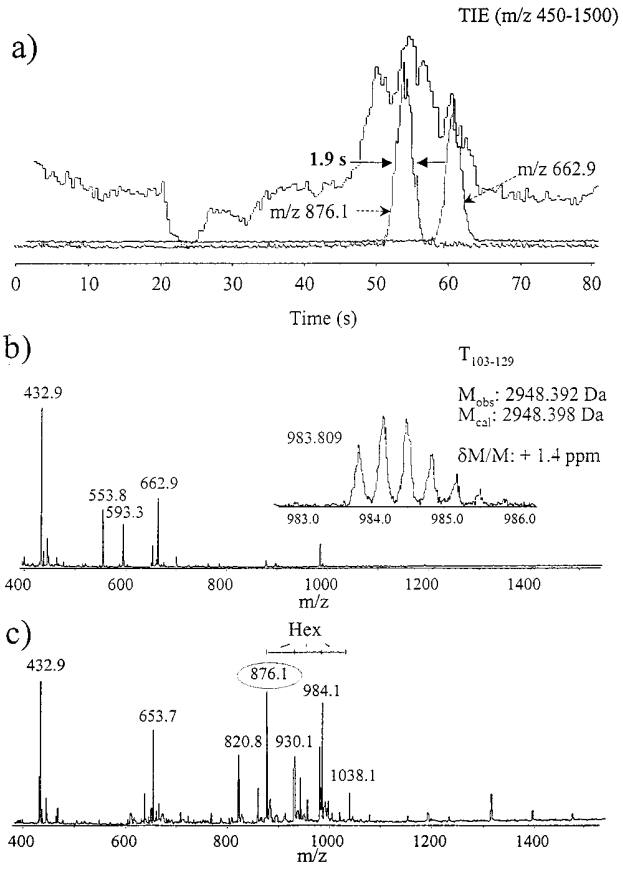

Figure 3. Chip-CE-QqTOF MS analysis of tryptic peptides from the seed lectin PHA-L: (a) TIE (m/z 400−1500) and RIE for m/z 662.9 and 876.1; (b, c) extracted mass spectra taken at (b) 53 s and (c) 61s. Conditions were as for Figure 2 except for a 30 s injection at −2.5 kV potential resulting in the injection of 2 nL of sample. A nanoelectrospray voltage of +2kV was applied at the BCQ/gold-coated emitter.

In the present experiment, a side channel port (well D in Figure 1) was filled with a dilute solution of angiotensin I. The calculated triply and doubly charged ions of angiotensin I (m/z 432.9003 and 648.8466) were used as internal references to measure the molecular masses of unknown peptides. An example of this is shown in Figure 3b for the extracted mass spectrum of the peak identified at 61 s in Figure 3a. One of the peptides

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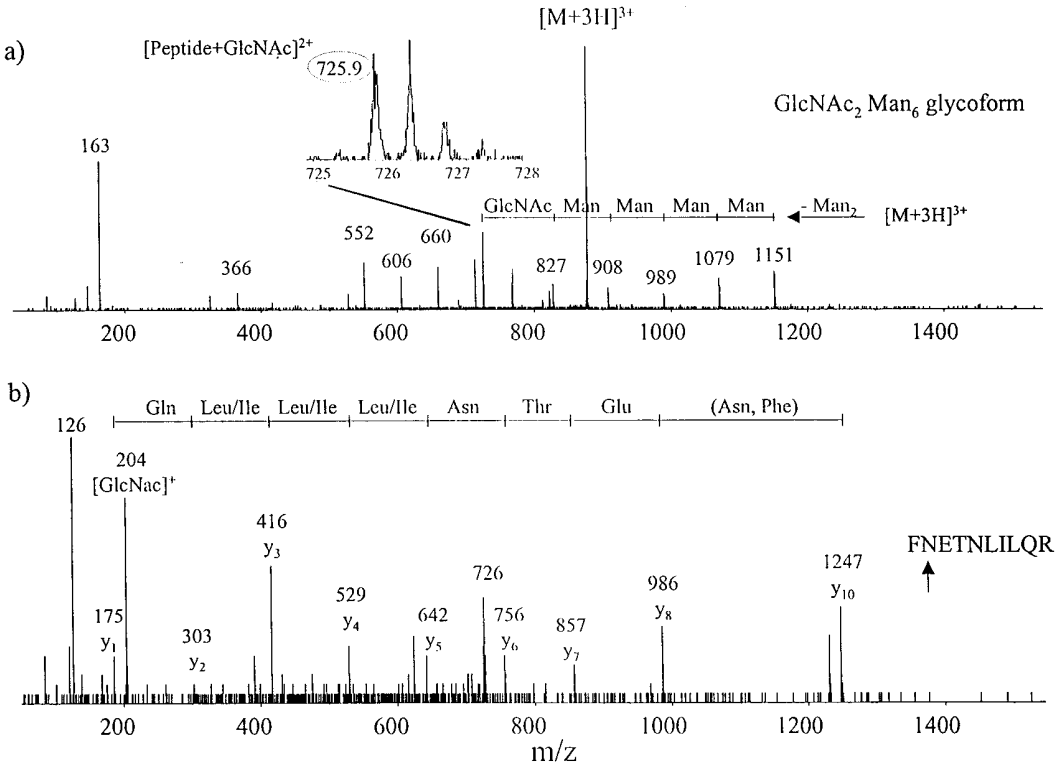

Figure 4. Chip-CE-MS-MS analysis of tryptic peptides from PHA-L: (a) product ion of *m/z* 876.1 and (b) second generation of fragment ion for precursor at *m/z* 726. Separation conditions were as for Figure 3 except that Ar was used as a target gas at a collision energy, E_{lab} (laboratory frame of reference), of 60 eV (orifice voltage 60 V) for (a) and 70 eV (orifice voltage 150 V) for (b).

observed corresponds to the predicted tryptic fragment $T_{103-129}$ as indicated by the triply charged ion at *m/z* 983.8 shown as an inset in Figure 3b. The instrument resolution was approximately 10 000 (peak width half-height definition), which enabled separation of isotopic components from this multiply protonated ion. The observed mass for this tryptic peptide was only 2 ppm off the calculated molecular mass derived from the protein sequence.[33] Accurate mass measurements obtained for the most prominent tryptic peptides observed in Figure 3 were used to determine the overall mass accuracy, and these results are summarized in Table 2. In all cases, the observed values were within 5 ppm of the calculated molecular masses. The precision and mass accuracy obtainable with this instrument could be of practical value for protein identification approaches using peptide mass fingerprinting. Indeed, with a mass tolerance of 10 ppm only three tryptic peptides of masses 1105.532, 1323.684, and 2948.398 Da (Figure 3b) were sufficient to uniquely identify this lectin (accession no. P05087) in a database search using the PeptideSearch algorithm.

It is noteworthy that the analysis presented in Figure 3 also shows the presence of tryptic peptide glycoforms, as evidenced by a series of triply charged ions separated by 54 *m/z* units in the extracted mass spectrum of the peak observed at 53 s (Figure 3c). Confirmation of this proposal was obtained by conducting on-line MS-MS experiments of selected precursor ions from a second sample injection. An example of this is presented in Figure 4 a for the triply protonated molecule at *m/z* 876.

The precursor ion was tentatively assigned as the doubly protonated glycopeptide $T_{11-20}GlcNAc_2Man_6$. This glycoform assignment was supported by the observation of the successive losses of mannose residues from the $[M + 3H]^{3+}$ precursor ion affording the doubly charged fragment ions extending from *m/z* 827 to *m/z* 1151. Consistent with previous MS-MS investigations on glycopeptides, this low energy MS-MS spectrum is dominated by cleavages of glycosidic bonds from the oligosaccharide side chain, with no fragmentation of the peptide amide bonds.[26,27] Peptide backbone sequence information was obtained by selecting the precursor ion at *m/z* 725.9 corresponding to the doubly charged fragment ion $[T_{11-20}GlcNAc\ ]^{2+}$ formed in the orifice/skimmer region of the QqTOF instrument by raising the orifice voltage to approximately 150 V (Figure 4b). The second generation

Table 2. Assignments of Proteolytic Fragments from a Tryptic Digest of *P. vulgaris* L Lectin

time (s)	M_{obs} (Da)	M_{calc} (Da)	error (ppm)	sequence
54.20	2625.139	2625.143	1.5	(R)FNETNLILQR(D)[a]
55.57	1958.026	1958.032	3.1	(K)LSDGTTSEGLNLANLVLNK(I)
56.54	1991.030	1991.036	3.0	(K)SVLPEWVSVGFSATTGINK(G)
57.01	1752.826	1752.832	3.4	(K)GNVETNDVLSWSFASK(L)
60.25	1323.684	1323.692	4.5	(K)TSFIVSDTVDLK(S)
60.30	2948.392	2948.398	1.4	(K)GGFLGLFDGSNSNFHTVA-VEFDTLYNK(D)
60.95	1105.532	1105.536	3.6	(R)DASVSSSGQLR(L)
62.10	1184.574	1184.578	3.4	(R)LTNLDGNGEPR(V)[b]
63.40	1183.590	1183.594	3.4	(R)LTNLNGNGEPR(V)
63.95	917.386	917.388	2.2	(K)DWDPTER(H)

[a] $Man_6GlcNAc_2$ glycoform appended on first Asn residue. [b] Deamidation product where a single Asn was converted to an Asp residue.

(33) Hoffman, L. M.; Donaldson, D. D. *EMBO J.* **1985**, *4*, 883−889.

fragment ions thus obtained are typical of tryptic peptides in that the spectrum is dominated by cleavage of the amide bonds yielding abundant y-type fragment ions from which the peptide sequence can be deduced. On the basis of these tandem mass spectrometric data and the presence of a consensus amino acid triad Asn-Glu-Thr, the site of attachment of this high mannose oligosaccharide was assumed to be Asn_{12} rather than Asn_{15}. It is noteworthy that this glycopeptide displays a glycoform distribution extending from six to nine mannose residues, similar to that observed previously for a close relative, *P. vulgaris* L lectin.[26]

Protein Identification of Gel-Separated Bands from Membrane Extracts of *H. influenzae*. The applicability of this technique to the identification of unknown proteins isolated from gel electrophoresis was evaluated for extracts of membrane components from the Rd and Eagan strains of *H. influenzae*. The Rd strain is an avirulent pathogen corresponding to that sequenced previously by Fleishman et al. in 1995,[34] while the Eagan strain is a serotype b clinical isolate associated with invasive infections of the blood stream, including septicemia, meningitis, and pneumonia.[35] Comparison of surface-associated proteins obtained from these two strains provides a basis for the development of vaccines against the *H. influenzae* pathogen.[36]

Separation of membrane proteins using two-dimensional gel electrophoresis is often precluded by the appearance of trailing bands in the isoelectric focusing dimension partly due to the noncovalent attachment of lipids and the precipitation of protein in the immobilized pH gradient strips. Despite recent advances using novel nonionic detergents and reducing agents to solubilize membrane-associated proteins, separation of the most hydrophobic components is typically conducted using one-dimensional SDS-PAGE (1D gel). An example of 1D-gel separation of membrane proteins from *H. influenzae* is presented in Figure 5 for both Rd and Eagan strains. For each strain, three loadings of approximately 0.5, 1, and 2 μg of membrane extract were applied on top of the gel. Discrete bands visualized by colloidal silver stains were then excised, the proteins were reduced and alkylated and subsequently digested with trypsin, and the resulting peptides were extracted from the gel pieces.

As a sensitivity test of the present approach, the chip-CE-nESMS analysis of tryptic peptides from spot 2, a weak band estimated to represent less than 25 ng of the original protein, is shown in Figure 6. The TIE of the corresponding analysis is presented in Figure 6a, along with the RIE for the selected peptide ion at m/z 600.4 The extracted mass spectrum for the m/z 600.4 ion is shown in Figure 6b and enabled the identification of this tryptic peptide as a doubly protonated ion. In a subsequent chip-CE-MS-MS experiment, this doubly protonated peptide was

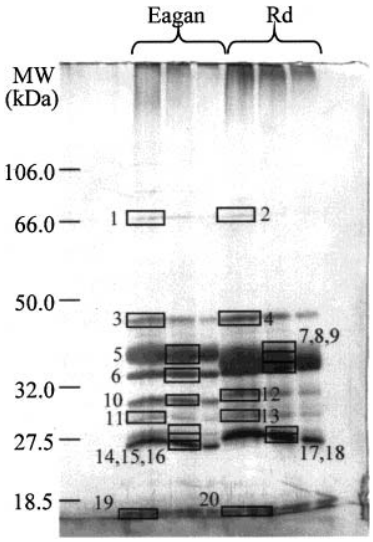

Figure 5. 1D-gel electrophoresis separation of membrane proteins from *H. influenzae* Rd and Eagan strains. Band annotations correspond to proteins identified in Table 3.

selected as a precursor ion; the resulting fragment ion spectrum is presented in Figure 6c. Despite the low intensity of the m/z 600.4 peak, the corresponding MS-MS spectrum provided valuable structural information on this trace-level protein. The tandem mass spectrometric analysis of the precursor ion at m/z 600.4 gave several singly charged y-type fragment ions from which the amino acid string (EF) could be deduced. This sequence segment plus the accurate molecular mass was sufficient to uniquely identify this band as the protective surface antigen D151 or D152 from *H. influenzae* although no restriction on the target organism was made in the database search. It is noteworthy that both protein candidates have highly homologous sequences with calculated molecular masses of 87.5 and 87.7 kDa, consistent with that expected for the 1D gel shown in Figure 5.

Similarly, the identity of each band identified in the 1D gel was established using both peptide mass fingerprinting and sequence tag approaches. The latter approach was deemed necessary in situations where ambiguities in the assignment of protein candidates from the mass fingerprinting database searching were found or when the peptide signal was relatively weak as shown in Figure 6. Misidentification of protein bands can also result from modifications of the original proteins or the occurrence of genetic variants comprising a number of substituted amino acids. Obviously, such structural changes would provide a number of different tryptic masses compared to those expected from the database protein sequence and care has to be exercised when one interprets data obtained from peptide-fingerprinting searches. Tandem mass spectrometry approaches to peptide sequencing can thus facilitate the characterization of a modification site while simultaneously providing low probability of false positive identification.

An example of a simple modification observed in membrane proteins from the *H. influenzae* Eagan strain is shown in Figure 7 for the band 5. The extracted mass spectra taken at 53 and 56 s from the same analysis are presented in Figure 7b,c, respectively. Different tryptic masses including 1007.50, 1027.50, 1219.60, 1312.70, 1396.74, 1540.66, 1643.94, 1690.84, 1745.86, 2804.88, and

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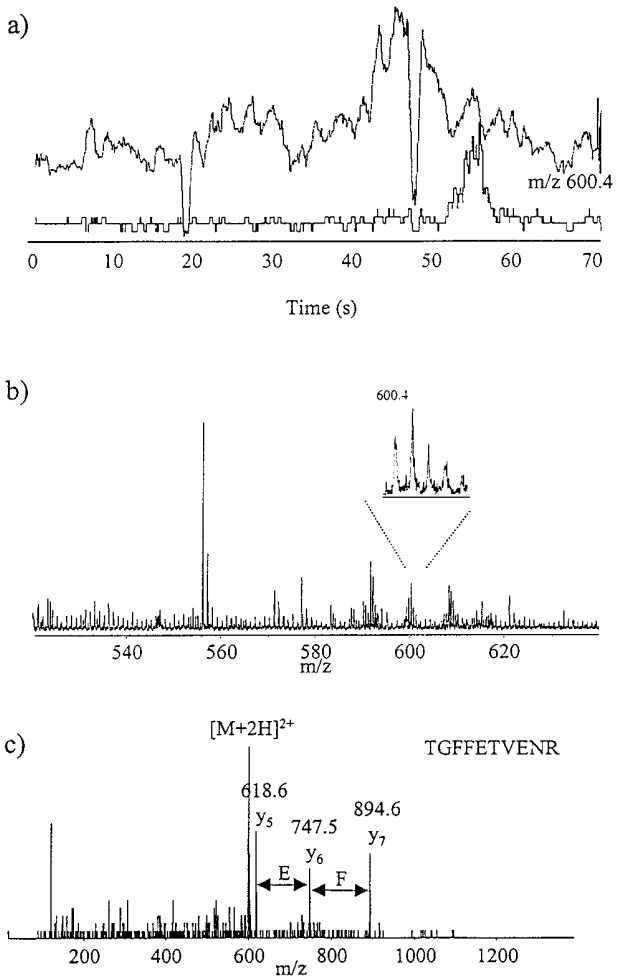

Figure 6. Chip-CE-QqTOF MS analysis of tryptic peptides from the membrane extract of *H. influenzae*, band 2 in Figure 5. Separation conditions were as for Figure 3: (a) total ion electropherogram (*m*/*z* 450−1500) and RIE for the *m*/*z* 600.4 ion; (b) extracted mass spectrum for the peak at 55 s; (c) MS-MS spectrum of the *m*/*z* 600.4 ion obtained from a separate injection. Conditions were as for Figure 4 except that a collision energy of 50 eV was used for (c).

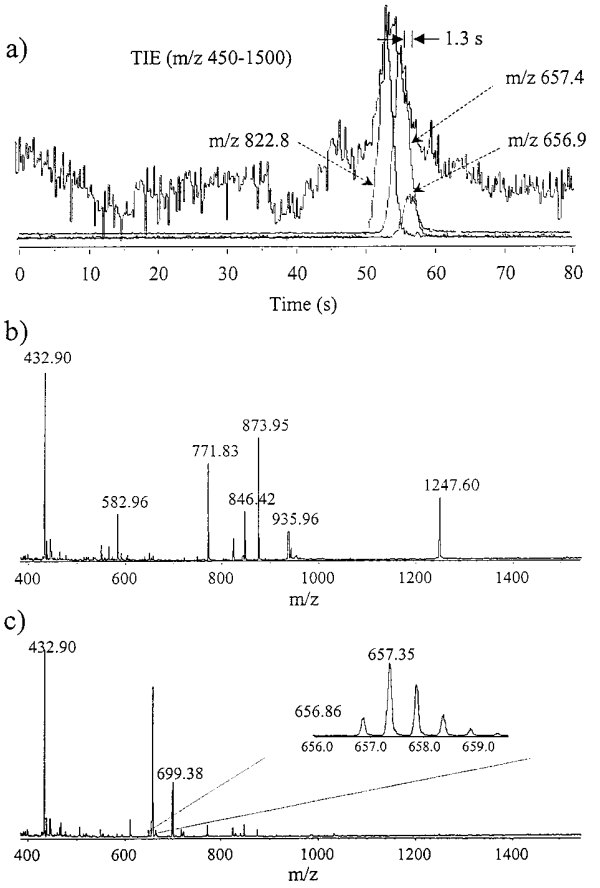

Figure 7. Chip-CE-QqTOF MS analysis of tryptic peptides from the membrane extract of *H. influenzae*, band 5 identified in Figure 5: (a) TIE (*m*/*z* 450−1500) and RIE for *m*/*z* 656.9, 657.4, and 822.9 ions; (b, c) extracted mass spectra for peaks migrating at (b) 53.0 and (c) 55 s. Separation conditions were as for Figure 3.

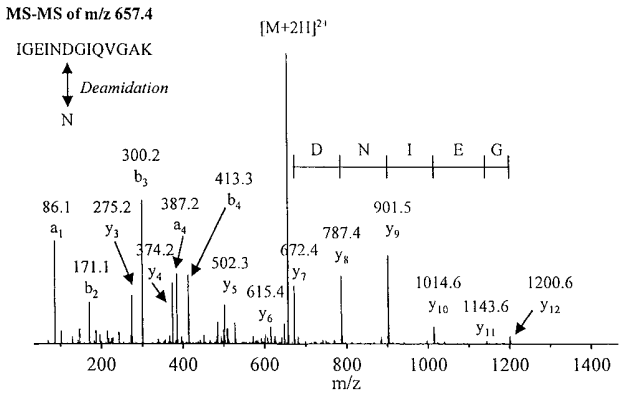

Figure 8. Product ion spectrum of the *m*/*z* 657.4 precursor identified in Figure 7c. Separation conditions were as for Figure 4 except that a collision energy, E_{lab}, of 60 eV was used.

3739.80 Da were used as an initial screen to identify potential protein candidates. The first protein candidate encoding the highest number of peptide masses (9 hits out of 13 masses) was the outer membrane protein OM21 (accession no. P20149). However, a few tryptic peptides such as that of mass 1312.70 Da (Figure 7c) could not be matched to the original protein, and further structural investigations were undertaken to identify the source of this discrepancy.

The MS-MS spectrum of *m*/*z* 657.4 is shown in Figure 8 with a partial sequence assignment derived from a series of y-type fragment ions extending above the *m*/*z* value of the precursor ion. The amino acid sequence for this tryptic peptide was consistent with a deamidation product resulting in a mass shift of 1 Da from the mass of the expected peptide IGEIN***N***GIQVGAK. The site of deamidation was assigned to the second asparagine residue based on the observation of a fragment ion at *m*/*z* 672.4 (Figure 8). Nonenzymatic deamidation of asparagine (and to a lesser extent on the glutamine residue) can occur naturally through isomerization and racemization from aspartyl and asparaginyl peptides under physiological conditions.[37] Structural determinants such as the presence of the C-terminal amino acids glycine and serine adjacent to the asparagine residue were found to accelerate this process.[38] A closer examination of Figure 7 enabled the identification of both aspartyl and asparaginyl tryptic peptides eluting at 55.0 and 56.3 s, respectively.

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Table 3. Assignments of Bands Obtained from 1D-Gel Separation of Membrane-Associated Proteins from *H. influenzae* (Figure 6)

band nos.	tryptic peptide masses[a]	protein candidates	mass (kDa)
1, 2	472.30, 915.50, 1045.64, 1060.68, 1173.82, *1198.82*, 1214.86, 1381.04, 1397.06, 1455.95, 1636.92	protective surface antigen D151 (P44935) protective surface antigen D152 (P46024)	87.5 87.7
3, 4, 10, 12	979.56, 1108.82, 1121.68, 1131.72, 1188.72, *1278.78*, *1486.84*, *1505.90*, 1542.94, 1569.84, 1606.94, 1622.84, 1700.06, 2159.28	outer membrane proteins OM11 (P43838) outer membrane proteins OM12 (P10641)	49.5 50.0
5, 7, 8	1007.50, 1027.50, 1219.60, *1312.70*, 1396.74, 1432.74, *1540.66*, *1643.94*, *1690.84*, *1745.86*, *2045.00*, 2804.88, 3739.80	outer membrane protein OM21 (P43839) outer membrane protein OM22 (P20149)	39.3 39.7
6, 9, 15, 16, 17, 18	930.62, 949.52, 1107.58, 1157.00, 1183.56, 1242.62, *1279.68*, *1527.72*, 1532.80, *1625.92*, 2032.20, *2392.16*, 2829.51, 3739.88	outer membrane protein OM51 (P38368) outer membrane protein OM52 (P43840)	37.6 37.7
11, 13	905.54, 950.62, 1010.70, 1090.76, 1147.76, 1505.92, *1696.10*, 1706.94	50S ribosomal protein L2; GN (P44343)	30.0
14	897.5, 1005.62, *1101.64*, 1182.80, 1745.07	outer membrane protein OM4 (P26093)	30.4
19, 20	535.37, *1130.68*, 1252.92, *1317.84*, 1626.08, *2420.44*, 2430.48	outer membrane protein OM6 (P10324)	16.1

[a] Masses in italics indicate tryptic peptides identified by on-line MS-MS.

A combination of tryptic peptide masses and partial sequence information was thus used to identify the different proteins observed in the 1D gel of membrane extracts for both Rd and Eagan strains of *H. influenzae* (Figure 5); a summary of the band assignment is presented in Table 3. Consistent with previous investigations, the porin OM21 and OM22 were found to be the most abundant outer membrane proteins in both *H. influenzae* Rd and Eagan strains.[36,39] This investigation also highlighted the presence of other important membrane components such as OM11 and OM12, which are known to play an important role in the passive transport of small molecules. It is noteworthy that, in some situations, a given protein candidate was found at two different apparent molecular masses on the 1D gel (Figure 5). This is indeed the case for the outer membrane proteins OM11 and OM12 (bands 3, 4, 10, and 12) together with OM 51 and OM52 (bands 6, 9, 15−18). While some protein heterogeneity can partly explain the occurrence of related bands close to those of the expected gene product, the reason for the observation of two bands for the same protein spaced by at least 20 kDa, as for OM11 and OM12, is still unclear. However, such observations were also noted for OM22 in previous investigations on the *H. influenzae* proteome using an LC/MS-MS approach.[40]

CONCLUSION

The coupling of modified microchip devices to BCQ/gold-coated nanoelectrospray emitters offers a novel and efficient sample introduction interface to mass spectrometry, especially when higher sensitivity and separation efficiency are required. The modification of both chip channel and nanoelectrospray emitter with BCQ coating imparts a positive charge on the silica surface, which minimizes peptide adsorption when acidic electrolytes are used. Peptide separations were typically conducted in less than 90 s with peak widths of approximately 2 s. The throughput for the analysis of peptide digests was limited by the speed at which the next sample could be loaded onto the chip (approximately 5 min). However, alternate chip designs now offer multiple wells for the introduction of separate solutions.

The temporal and mass spectral resolution available on these devices enabled the analysis of closely related peptides such as protein deamidation products, where the aspartyl and asparaginyl peptides are separated by 1.3 s. Low-nanomolar detection limits were obtained for different peptides and for in-gel digests of proteins. Small sample reservoirs were integrated on the chip device and enabled the loading of approximately 4 μL of the tryptic digest solution. Further refinements of the sample introduction system should provide the application of smaller sample volumes though evaporation could be a limiting factor to the success of the analysis. The combination of an efficient separation device with a high-speed data acquisition hybrid quadrupole/time-of-flight mass spectrometer offers new possibilities for rapid identification of trace-level proteins.

The application of the chip-CE-QqTOF MS system was demonstrated for the analysis of glycoproteins and the identification of gel-separated membrane proteins from extracts of different *H. influenzae* strains. Accurate mass measurements of migrating peptides were obtained using an internal standard infused directly from a side channel of the chip device. These measurements facilitated protein identification using peptide mass fingerprinting and database searching. In situations where higher levels of specificity are required (e.g., the presence of more than one protein/band), peptide sequence information can be obtained by on-line tandem mass spectrometry of previously identified precursor ions. Although this process required a second sample injection, software developments are currently in progress to facilitate the incorporation of data-dependent acquisition. This report also highlights the fact that the coupling of the chip-CE-QqTOF MS provided an efficient method for the identification of trace-level proteins present at less than 50 ng in gels without any additional sample cleanup and/or on-chip preconcentration. While this approach is limited to methods in which the size of the proteome evaluated can be reduced as it was here, it illustrates that the chip-CE-nESMS device interfaced to a QqTOF mass spectrometer can play a role in increasing the levels of automation available for rapid analysis of the proteome.

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membrane proteins, and T. Cassidy (NRC, INMS) for the gold plating of nanoelectrospray emitters. This work was funded through the National Research Council (NRC)−Natural Sciences and Engineering Research Council of Canada (NSERC) partnership program with the industry support of Perkin-Elmer/Sciex. We thank the Alberta Microelectronics Corp. for device fabrication. This is an NRCC contribution no. 42402.

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