Application of Microfluidic Devices to Proteomics Research

IDENTIFICATION OF TRACE-LEVEL PROTEIN DIGESTS AND AFFINITY CAPTURE OF TARGET PEPTIDES

Jianjun Li‡, Tammy LeRiche‡, Tammy-Lynn Tremblay‡, Can Wang§, Eric Bonneil‡¶, D. Jed Harrison§, and Pierre Thibault‡

This report describes an integrated and modular microsystem providing rapid analyses of trace-level tryptic digests for proteomics applications. This microsystem includes an autosampler, a microfabricated device comprising a large channel (2.4 μl total volume), an array of separation channels, together with a low dead volume enabling the interface to nanoelectrospray mass spectrometry. The large channel of this microfluidic device provides a convenient platform to integrate C18 reverse phase packing or other type of affinity media such as immobilized antibodies or immobilized metal affinity chromatography beads thus enabling affinity selection of target peptides prior to electrophoretic separation and mass spectrometry analyses on a quadrupole/time-of-flight instrument. Sequential injection, preconcentration, and separation of peptide standards and tryptic digests are achieved with a throughput of up to 12 samples/per h and a concentration detection limit of ~5 fmol (25 fmol on chip). Replicate injections of peptide mixtures indicate that reproducibility of migration time was 1.2–1.8%, whereas relative standard deviation ranging from 9.2 to 11.8% are observed on peak heights. The application of this device for trace-level protein identification is demonstrated for two-dimensional gel spots obtained from extracts of human prostatic cancer cells (LNCap) using both peptide mass-fingerprint data base searching and on-line tandem mass spectrometry. Enrichment of target peptides prior to mass spectral analyses is achieved using c-myc-specific antibodies immobilized on protein G-Sepharose beads and facilitates the identification of antigenic peptides spiked at a level of 20 ng/ml in human plasma. Affinity selection is also demonstrated for gel-isolated protein bands where tryptic phosphopeptides are captured on immobilized metal affinity chromatography beads and subsequently separated and characterized on this microfluidic system. Molecular & Cellular Proteomics 1:157–168, 2002.

Proteomics research entails the global characterization of proteins expressed in cells under defined conditions. Such studies are particularly important in view of the conflicting evidence regarding the correlation between the abundance of expressed proteins and gene-expression levels obtained from mRNA microarrays (1, 2). The monitoring of protein expression profiles remains a very challenging task because of the wide dynamic range of expressed proteins and the variability of gene products (splicing variants, N- and C-terminal truncations, co- and post-translational modifications, etc.), which may change between and within the tissues of an organism (3). The traditional approach to isolating and characterizing proteins from biological samples has been separation by two-dimensional gel electrophoresis (2-D) gel, followed by identification of protein spots using sensitive mass spectrometry techniques and data base searching (3–5). An alternate approach to 2-D gel includes a comprehensive chromatographic separation of the proteolytic fragments derived from intact proteins, followed by mass spectral identification and data base searching. This can be achieved using a two-dimensional liquid chromatography approach whereby peptides are fractionated on a strong cation exchange column, followed by an extended gradient elution on a C18 reverse phase column (6, 7). This two-dimensional chromatography approach has been described recently for the comprehensive identification of the yeast proteome (6,113 proteins) and provided the identification of 1484 proteins among which were 131 proteins with three or more predicted transmembrane domains (8). Although questions remain concerning the ability of the 2-D gel approach to analyze hydrophobic proteins and to quantitate and characterize the full dynamic range of protein expression from a given genome (9), protein identification via 2-D gel and mass spectrometry is still widely used in numerous proteomics core facilities. This approach enables the visualization of a very large number of proteins simultaneously, and in contrast to 2-D chromatographic separation it facilitates the identification of post-translational modifications and proteo-

1 The abbreviations used are: 2-D, two-dimensional; 2-D gel, 2-D gel electrophoresis; MS-MS, tandem mass spectrometry; LC, liquid chromatography; BCQ, [acryloylamino]propyltrimethylammonium chloride; TEMED, N,N,N',N‘-tetramethylmethylenediamine; DMP, dimethylpimelimidate; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid; RI E, reconstructed ion electrophoregram(s); IMAC, immobilized metal affinity chromatography; CE, capillary electrophoresis; nESMS, nanoelectrospray mass spectrometry.

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lytic processing in a convenient reference format. Furthermore, differential protein expression profiles can be compared easily for large data sets and for protein extracts obtained under different growth conditions, biological perturbations, or following pre-fractionation through organelle-enrichment methods.

Unambiguous identification of gel-isolated proteins typically relies on sensitive tandem mass spectrometry (MS-MS) techniques to obtain partial amino acid sequences, which in combination with the mass of the precursor ion and that of the unidentified N- and C-terminal segments, can be used to search protein data bases (10–12). The combination of microscale LC column (50–180 μm inner diameter column) to nanoelectrospray MS-MS has also become a widely used workhorse system for proteomics applications involving the identification of 50–100 fmol of in-gel digests with a duty cycle of <15 min/sample (13, 14). The coupling of microfluidic devices to mass spectrometry also offers an efficient means of handling small liquid volumes while simultaneously performing separation and sensitive detection on devices of small footprint. These microfabricated devices do not involve moving parts or high pressure liquid chromatography pumps as the analysis format involved in the precise control of voltages and electrical fields across small separation channels. The microchip interface to mass spectrometry has been achieved by creating a guided channel to the nanoelectrospray emitter using butted capillary (15), the double-etching procedure (16), polymer casting (17), and microdrilling (18, 19). Rapid separations on these microfluidic devices have been demonstrated using time-of-flight (20–22) and ion trap (15, 16) mass analyzers for submicromolar sample concentrations (15, 17, 21). On-line stacking or adsorption preconcentration can also be applied prior to electrophoretic separation to enhance sensitivity (23). Obviously, significant advances in automation are still needed to provide the required throughput and ruggedness sought for the rapid and positive identification of proteins. To this end, efforts are also devoted to microfabricated chip designs that enable sample introduction from autosampler or microwell plates (24, 25).

In an effort to accelerate the analysis of protein digests while simultaneously maintaining the flexibility of the current microfluidic device, the present investigation describes the integration of an autosampler coupled to the chip via a convenient sample port. The previous chip design (21, 25) was modified to include a port of low flow resistance enabling the introduction of sample on and off the chip without perturbing the fluids in the analysis manifold. Such design was used previously to conduct on-chip tryptic digestion and provided a convenient format to conduct rapid in-solution digest (10–15 min) (26). In the present investigation we have used the large channel of this microfluidic to integrate C18 reverse phase packing or other types of affinity selection media to select target peptides prior to CE separation and mass spectrometry analyses. Application of this integrated system is demonstrated for the analysis of trace-level tryptic peptides obtained from gel-isolated proteins of human prostatic cell extracts.

**EXPERIMENTAL PROCEDURES**

**Material and Chemicals**—Fused-silica capillary was purchased from Polymicro Technologies (Phoenix, AZ), and Teflon tubing was from LC Packing (San Francisco, CA). Peptides and protein standards were purchased from Sigma and used without further purification. Gold-electroplating solution was prepared with 24K Bright English gold Plating Salts (Grobet File Co. of America, Inc., Carisbad, 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, and formic acid was from BDH Inc. (Toronto, ON, Canada). Dimethyl pimelimidate (DMP), ethanamine, and sodium azide used for antibody coupling were obtained from Aldrich. The C18 reverse phase packing material of 40 μm was excised from Sep-Pak cartridges obtained from Waters (Milford, MA), and 5 μm Poros was from Applied Biosystems (Framingham, MA). IMAC beads were purchased from Amersham Biosciences, Inc.

**Preparation of Immunoaffinity Selection Media**—Mouse ascites fluid containing the monoclonal anti-c-myc (mouse IgG, isotype) was obtained from Dr. R. MacKenzie (Institute for Biological Sciences, Ottawa, Ontario, Canada). The ascites fluid was diluted 3-fold in 20 mM phosphate buffer (pH 7) and centrifuged at 10,000 rpm for 20 min to remove lipids. The supernatant was purified using a Hitrap protein A column (Amersham Biosciences, Inc.) under isocratic elution using 0.1 M citric acid (pH 3). The UV absorbance was monitored at 280 nm. The antibody fraction was adjusted to pH 6 using 0.2 M Tris buffer (pH 8) and dialyzed against phosphate-buffered saline. The antibody was cross-linked to protein G-Sepharose 4 fast flow gel (Amersham Biosciences, Inc.) using DMP. Briefly, 2 mg of IgG, antibody was added to 1 ml of the protein G gel and incubated for 1 h at room temperature with occasional mild shaking on a vortex. A DMP solution (in 0.2 M sodium borate) was added to the protein G-bound antibody gel slurry to a final concentration of 20 mM borate and incubated at room temperature for 30 min. The DMP solution was removed, the gel was washed once with borate solution and resuspended in 0.2 M ethanolamine (pH 8) for a 2-h incubation period. The ethanolamine solution was replaced by 0.02% NaN3 in phosphate-buffered saline and stored at 4°C prior to use.

**Cell Cultures**—Human prostatic cancer LNCap cells (CRL-1740) were obtained from the American Type Culture Collection (Manassas, VA). LNCap cells were grown in RPMI 1640 medium supplemented with 8% fetal bovine serum (Sigma) at 37°C and 8% carbon dioxide. Cells were harvested by gentle scraping and washed three times in phosphate-buffered saline at 4°C. Cell pellets were then frozen at −80°C until further processing.

**Protein Isolation and Purification**—LNCap cells were resuspended in lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 1% dithiothreitol). After shaking at room temperature for 1 h, the lysate was centrifuged for 10 min at 12,000 × g to pellet unbroken cells and nuclei. Protein content in supernatant was then evaluated by Bradford assay.

**Gel Electrophoresis**—Samples containing 300 μg of total cell lysate was used to rehydrate immobilized pH gradient strips (pH 5–8) (Bio-Rad, Hercules, CA). First dimension electrophoresis was performed using the following program: 200-V rapid ramp for 1 h, 500-V rapid ramp for 1 h, 5000-V linear ramp for 5 h, 5000-V focusing for 8000 Vh. Prior to second dimension electrophoresis, proteins on immobilized pH gradient (IPG) strips were reduced (1% dithiothreitol) and alkylated (4% iodoacetamide) in SDS equilibration buffer (6 M urea, 30% glycerol, 2% SDS, 50 mM Tris-HCl (pH 8.8)). The second dimension is performed on a 10% gel (1-mm thick) at a constant 24 mA per
gel. Separated proteins were then fixed in the gel using 50% ethanol, 5% acetic acid, stained with silver nitrate, and scanned using the Fluo-S imager (Bio-Rad).

**Protein Digestion**—Selected spots were excised manually under a laminar flow hood. Excised spots were placed in a 96-well plate with a pierced-well bottom. Protein spots were processed using a Progenesis automated digestion unit (Genomics Solutions, Ann Arbor, MI). Briefly the procedure involved spots destaining with potassium ferricyanide solution (15 mM potassium ferricyanide, 50 mM sodium thioglu-
sulfate). Gel pieces were then rinsed three times with water and shrunk with acetonitrile. The gel pieces were reswelled with 10 to 20 
µl of trypsin solution (0.01 µg/µl in 50 mM ammonium carbonate), and 30–50 
µl of 50 mM ammonium carbonate is added to the gel pieces prior to overnight incubation (37 °C) with trypsin (Promega). Digestion solution containing peptide fragments was combined with organic extracts (30–50 
µl of 50% methanol, 5% acetic acid) of the gel pieces. Peptide extracts were then evaporated to dryness on a Savant preconcentrator.

**Device Fabrication, BCQ Coating, and System Integration**—The microfluidic device shown in Fig. 1a was fabricated at the University of Alberta Microfab laboratory (Edmonton, Alberta, Canada), as de-
described previously (25, 26). Channels were etched on Corning 0211 glass (Corning Glass) using standard photolithography and wet chemical etching techniques. Channels were etched on one glass plate to a 10-µm depth and 30-µm-width for the separation channels, with 230-µm-wide segments near the reservoirs. Channel lengths were essentially the same as PCRD2, described previously (21). The injector to capillary distance was 45 mm, the double-T injector had a 100-µm offset, and the additional channel attached to reservoir D was 24 mm from the junction. A large volume channel, 300-µm-wide, 150-µm-deep, and 22-mm-long, was etched into the cover plate, after which access holes were drilled into the cover hole. The channels on the chip were covalently modified with BCQ coating prior to inserting the nanoelectrospray emitter into a flat-bottom hole at the exit of the separation channel (21). Small plastic pipette tubes were inserted through small holes made in the center of the septa (Thermo
green LB-1 from Supelco or Septa 77 from Chromatographic Specialties) for buffer (B) and waste (A) reservoirs. Teflon tubes (180-µm inner diameter) were inserted in 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 seal a capillary transfer line. Channels were etched on one glass plate to a 10-µm-depth and 30-µm-width for the separation channels, with 230-µm-wide segments near the reservoirs. Channel lengths were essentially the same as PCRD2, described previously (21). The injector to capillary distance was 45 mm, the double-T injector had a 100-µm offset, and the additional channel attached to reservoir D was 24 mm from the junction. A large volume channel, 300-µm-wide, 150-µm-deep, and 22-mm-long, was etched into the cover plate, after which access holes were drilled into the cover hole. The channels on the chip were covalently modified with BCQ coating prior to inserting the nanoelectrospray emitter into a flat-bottom hole at the exit of the separation channel (21). Small plastic pipette tubes were inserted through small holes made in the center of the septa (Thermogreen LB-1 from Supelco or Septa 77 from Chromatographic Specialties) for buffer (B) and waste (A) reservoirs. Teflon tubes (180-µm inner diameter) were inserted in 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 air-tight seal between the sample/ buffer reservoirs and the chip device.

**Sequential Injection chip-CE-nESMS with Embedded Adsorption and Affinity Selection Media**—A 0.1 M formic acid solution was used for separation of background electrolytes. All aqueous solutions were filtered through a 0.45-µm filter (Millipore, Bedford, MA) before use. The separation and waste reservoirs were filled with 30 µl of running buffer. A custom-made 96-well plate autosampler comprising a sealed pressurized inlet (27) was used to introduce an 8–10-µl sample plug to the chip device via a 40-cm length of transfer capillary (360-µm outer diameter, 50-µm inner diameter). The first two rows of the plate were filled with wash and organic buffers whereas the remaining 72 wells were filled with reconstituted protein digests. Manual packing of the stationary phase or affinity selection media was achieved by loading the slurry (typically 10 µl of bead suspension) to well C using a syringe while a constant vacuum was applied to well D. For C18 reverse phase packing, a mixed bed composed of 2 mm of 40-µm beads (Waters) and ~18 mm of 5-µm Poros particles (Applied Biosystems) was filled in sequence into the large channel. The reverse phase slurry was prepared by suspending 10 mg of beads in 50 µl of methanol prior to packing on the chip. For affinity selection using IMAC the beads were washed using 1% acetic acid in 10% aqueous acetonitrile and conditioned with 3 × 10 
µl of an aqueous solution of 100 mM ferric chloride (Aldrich). The beads were washed again with 1% acetic acid in 10% aqueous acetonitrile, suspended in 50 
µl of the same solution, and applied to the large packing channel. The IMAC bed was conditioned with 20 
µl of 0.1 M formic acid before loading the sample on the chip. Prior to sample loading (20 
µl) on the chip, the sample was acidified by reconstituting the in-gel digest in 5% acetic acid for proper binding of phosphopeptides on IMAC medium. Following sample application, the beads were washed with deionized water, and the selected peptides were eluted from the beads using 2% ammonium hydroxide. To minimize bubble formation a short plug of deionized water (200 nl) was intercalated between the acidic and basic buffers and provided sample stacking. In all cases, a constant voltage of ~4.5 kV was applied to well D during the electrospray injection whereas other wells were floated.

**Mass Spectrometry**—Mass spectrometric experiments were conducted using a Q-Star quadrupole/time-of-flight instrument (MDS/ Sciex). The mass spectral resolution (half-height definition) was 10,000. The interface was optimized by infusing a solution of 1 
µg/ml of angiotensin I at a flow rate of 0.2 
µl/min through well E using a Harvard syringe pump. During the separation, this peptide solution was introduced at a flow rate of 50 nl/min, and the [M + 3H]3+ and [M+2H2O]2+ ions of angiotensin I were used as internal mass markers for accurate mass measurements. Tandem mass spectra were obtained using the Q-Star, and collisional activation of selected precursor ions was obtained using nitrogen as a target gas at collision energies of typically 50 to 90 eV (lab
coryme frame of reference). Fragment ions formed in the RF-only quadrupole were record by a time-of-flight mass analyzer.

**Data Base Searching with Mass Spectrometric Data**—Accurate peptide masses were determined using internal standardization on the Q-Star instrument and were transferred to the ProteinProspector program. The list of peptide masses was searched against a nonredundant protein sequence data base from NCBI or SwissProt. Param-
ers for all searches assumed that masses corresponded to tryptic peptides and that cysteine residues were converted to S-
carbamidomethylcysteine. All peptide masses were considered monoisotopic, and the maximum deviation between the calculated and measured masses was set to <10 ppm. Alternatively, the search was conducted using the peptide sequence tag approach where the pre-
cise molecular mass of a given tryptic peptide plus the fragment ion m/z values derived from the MS-MS spectrum were used to retrieve potential protein candidates. In situations where no match was ob-
tained from either peptide mass fingerprinting or sequence tags, sequence segments of at least six amino acids were subjected to BLAST search at the NCBI web site (www.ncbi.nlm.nih.gov).

**RESULTS**

**Development of a Sequential Injection Chip Device with Embedded Adsorption Media**—In a previous report from this laboratory (25), we described the application of a modular microfabricated device comprising a relatively large sample introduction port (2.4 
µl total volume), an array of separation channels, together with a low dead volume, enabling the interface to nanoelectrospray mass spectrometry. When coupled to an autosampler, this system provided sequential injection (1.5 
µl per injection) and separation of in-gel tryptic digests with a throughput of up to 25 samples per hour with less than 3% sample carryover. The concentration detection limit for peptide analysis was in the low micromolar range comparable with microfluidic devices of similar configuration (21, 25). Although 1–2 
µl of sample was introduced to the large chip
channel the overall sensitivity of this approach was limited by the actual injection volume on the chip device (typically <5 nl). Consequently, this resulted in low sample utilization (0.2–0.3%) and was of limited practical use for excised spots obtained from 2-D gel electrophoresis where less than 1 pmol of gel-separated protein is typically available.

To improve the sensitivity of the present system, C18 adsorption beads were packed into the large chip channel. We also developed a pressurized inlet system configured to accept a 96-well plate, thus compatible with sample formats used for automated in-gel digestion and processing (27). This new device, which is shown schematically in Fig. 1a, enables the loading and adsorption of more than 10 μl of tryptic digest solution via a large channel of low resistance to flow (surface area 1.1 × 10^-3 cm^2) delimited by the inlet (C) and outlet (D) wells. Following sample processing, in-gel protein digestions result in 20 μl of final sample volume thus providing sufficient material for two separate injections should this be required. Also indicated in Fig. 1a is an injection channel of smaller dimension (surface area 2.6 × 10^-6 cm^2) that joins the sample introduction channel near well D. The dimensions of the separation channel were kept relatively small compared with the introduction channel (surface ratio of ~1:400 between the separation and introduction channel) to minimize the contamination of fluids between the two manifolds.

Adsorption preconcentration of sample prior to electrophoretic separation requires proper synchronization of sample loading and sample elution (Fig. 1b). The sequence steps leading to sample separation consist of injecting 8–10 μl of sample solution on the large chip channel, followed by 2 μl of wash buffer (0.1 M aqueous formic acid), 200 nl of desorption buffer (75% aqueous acetonitrile in 1% formic acid), and 1 μl of wash buffer (0.1 M aqueous formic acid). The sample is also dissolved in an acidic buffer (0.1 M HCOOH) to prevent any mismatch in solvent conductivity with the desorption and separation buffers. The sample flow rate through the chip channel is ~2 μl/min, and the entire cycle takes ~5 min/sample. In preliminary experiments, the arrival of the sample plug to the adsorption channel was carefully monitored to synchronize the necessary steps for sample loading, wash, and elution. The voltage of the nanoelectrospray emitter was maintained at +2.2 kV throughout the sample loading/desorption cycle whereas a voltage of -4.5 kV was applied at well D, and other wells were floated. This also avoided having to synchronize the electrokinetic injection with the arrival of the desorption plug to well D. The sample injection volume is ~10–20 nl, which in turn corresponds to a sample utilization of 5–10%. Experiments are presently underway to enhance sample loading by reducing the flow rate through the large channel following sample desorption.

The performance of this adsorption flow injection chip-CE-nESMS device with respect to sensitivity was first evaluated using a dilute solution of a Leu-enkephalin peptide standard (Fig. 2). Serial dilutions of peptide solutions ranging from 2 to 200 fmol were loaded on this chip-CE-nESMS device coupled to a quadrupole/time-of-flight instrument. The reconstructed ion electrophoregram (RIE) corresponding to Leu-enkephalin (m/z 556.3) is presented in Fig. 2. As indicated, a detection limit of 25 fmol (5 nl) was reached for a 5-μl injection. It is noteworthy that only 2.5 fmol is actually detected by the mass spectrometer considering a 10% sample utilization. Separate experiments conducted on a capillary LC-MS system (5-cm × 75-μm inner diameter column) fitted to a quadrupole/time-of-flight instrument yielded a limit of detection of 5 fmol for Leu-enkephalin for
a 10-µl sample injection (data not shown). The sensitivity of the present microfabricated chip-nESMS system is thus within a factor of 5 of that typically obtainable using a capillary LC-MS of similar configuration. This is also consistent with recent results describing a multiplex adsorption device for nanoelectrospray mass spectrometry where a detection limit of 8–80 fmol was obtained for a 5-µl injection (27). Further improvement in sensitivity and sample utilization is also expected from using more compact stationary bed and smaller desorption volumes than that typically obtained in the present manifold.

This system also afforded good linearity with respect to ion response and sample concentration, and regression coefficient, \( r^2 > 0.98 \), was obtained for all peptides examined. To assess the reproducibility of this autosampler system, replicate injections of 500 nM peptide solutions (250 fmol each injected on chip) were conducted in an uninterrupted fashion over a 25-min period (Fig. 3) using a limited time-of-flight mass range acquisition of \( m/z \) 500–800. Good reproducibility of migration times was obtained, with relative standard deviation values of 1.2–1.8%, whereas relative standard deviation ranging from 9.2 to 11.8% were observed on peak height consistent with earlier experiments obtained on the flow injection analysis chip-CE-nESMS system (25).

The application of this sequential injection chip device with imbedded adsorption channel was next tested using excised protein spots obtained from the 2-D gel separation of a total cell lysate from a prostatic cancer cell line LNCap. A 2-D gel corresponding to the separation of a 300-µg protein extract is shown in Fig. 4a and revealed more than 450 spots visible by silver staining. A small subset of 72 spots of different intensities was selected for excision and subsequently subjected to in-gel tryptic digestion (Fig. 4a). A microtiter plate comprising the 72 resuspended protein digests, together with wash and desorption buffers, was analyzed in an automated fashion using the adsorption preconcentration chip device. For each sample a total ion electropherogram was obtained for the individual tryptic peptide ions.

**Fig. 2.** Replicate injection of serial dilution of peptide standards (5–100 nm) obtained under full mass scan (\( m/z \) 400–1000). RIE for \( m/z \) 556.3. Inset shows the narrow mass spectrum for the MH\(^+\) ion of Leu-enkephalin obtained for the 5 nm injection. Conditions, sequential loading of peptide samples, followed by 200 nl of 1% formic acid in 75% acetonitrile. Electrokinetic injection and separation, −4.5 kV applied to well D whereas other wells are floated. A voltage of +2.2 kV was applied to the nanoelectrospray emitter.

**Fig. 3.** Replicate injections of 5 µl of three peptide mixtures present at 0.5 µM each. a, total ion electrophoregram (TIE) (\( m/z \) 400–1000) and RIE profiles for (b) \( m/z \) 556.3 and (c) \( m/z \) 523.8. Conditions were as for Fig. 2.
An example of this is shown in Fig. 4, b–d for the reconstructed ion electropherograms of two doubly charged peptide ions taken from each of spots 67–69. From these preliminary survey scans, a number of tryptic peptide ions were identified and subsequently subjected to data base search. To improve the confidence level in peptide mass fingerprinting experiments and to reduce the number of potential protein candidates, on-line accurate mass measurements for each tryptic peptides was achieved by introducing angiotensin I from well E in Fig. 1a. The calculated triply and doubly charged ions of angiotensin I (m/z 432.90033$^3$ and 648.84662$^2$, respectively) were then used as internal reference to measure the molecular masses of unknown peptides. Examination of the combined mass spectrum for each spot enabled the identification of several doubly protonated tryptic peptide ions such as m/z 600.3370, 606.3456, 627.3081, 744.3507, 830.4473, and 991.4927 for spot 69 (data not shown). None of these peptides were attributable to trypsin autolysis products and could be selected for data base search using peptide mass fingerprint (see “Experimental Procedures”). Results from this particular search supported the protein heat shock cognate 71-kDa protein as a potential protein candidate with a distinct MOWSE score of 926 (5/6 matches). The best protein match obtained for the three selected spots is shown under the corresponding electropherogram in Fig. 4, b–d.

The sensitivity of the present device also enabled protein identification via on-line tandem mass spectrometry and reduced ambiguity relating to false-positive matches sometimes found using peptide mass fingerprint. This application is shown in Fig. 5 for the identification of spot 14. The survey scan for this analysis (Fig. 5b) showed a doubly charged ion m/z 506.7, and the corresponding product ion spectrum (Fig. 5c) clearly indicated the sequence tag (554.3)FDP(913.4). Data base search using this information provided a single match to ubiquinol-cytochrome c reductase. Interestingly, the position of this protein on the 2-D gel was different from...
**TABLE I**

Identification of protein spots from the 2-D gel electrophoresis of total cell lysate of prostatic cancer cell line LNCaP (Fig. 4a)

| Spot | Protein or sequences | Molecular mass (Da)/pI | Accession number |
|------|----------------------|------------------------|------------------|
| 1    | Initiation factor 5A | 16.7/5.1               | P10159           |
| 2    | Superoxide dismutase | 15.8/6.0               | P00441           |
| 3    | Adenine phosphoribosyltransferase | 19.5/6.0 | P07741 |
| 4    | Thioredoxin peroxidase 1 | 21.9/5.9 | P32119 |
| 5    | ATP synthase D chain, mitochondrial | 18.4/5.3 | O75947 |
| 6    | Lactoylglutathione lyase | 20.6/5.3 | O04760 |
| 7    | Unmatched             |                        |                  |
| 8    | Apolipoprotein A-1 precursor | 30.8/5.7 | P02647 |
| 9    | Unmatched             |                        |                  |
| 10   | Unmatched             |                        |                  |
| 11   | Mitochondri thioredoxin-dependent peroxidin | 27.7/8.1 | P30048 |
| 12   | DJ-1 protein         | 19.8/6.7               | Q99497           |
| 13   | Lysophospholipase    | 24.7/6.7               | O75608           |
| 14   | Ubiquinol-cytochrome c reductase | 29.6/8.6 | P47985 |
| 15   | ESI protein homolog  | 26.1/8.5               | P30042           |
| 16   | Collagen α 3 chain   | 34.3/8.2               | P12111           |
| 17   | Triosephosphate isomerase | 26.5/6.9 | P00938 |
| 18   | Neurofibromin or its isoforms | 31.7/6.9 | P21359 |
| 19   | Antioxidant protein 2 | 24.9/6.3              | P30041           |
| 20   | Triosephosphate isomerase | 26.5/6.9 | P00938 |
| 21   | Antioxidant protein 2 | 24.9/6.3              | P30041           |
| 22   | Endoplasmic reticulum protein ERP29 | 29.0/7.4 | P30040 |
| 23   | Enoyl-CoA hydratase, mitochondrial | 31.4/8.5 | P30084 |
| 24   | Endoplasmic reticulum protein ERP29 | 29.0/7.4 | P30040 |
| 25   | Heat shock 27-kDa protein | 22.3/8.3 | P04792 |
| 26   | Prohibitin           | 29.8/5.7               | P35232           |
| 27   | Unmatched             |                        |                  |
| 28   | Chloride intracellular channel protein | 26.9/5.1 | O00299 |
| 29   | Annexin V            | 35.8/5.0               | P08758           |
| 30   | Unmatched             |                        |                  |
| 31   | Unmatched             |                        |                  |
| 32   | Unmatched             |                        |                  |
| 33   | L-lactate dehydrogenase H chain | 36.5/6.0 | P07195 |
| 34   | 60 S acidic ribosomal protein P0 | 34.3/5.9 | P05388 |
| 35   | Electron transfer flavoprotein α-subunit | 31.5/8.8 | P13804 |
| 36   | Electron transfer flavoprotein α-subunit | 31.5/8.8 | P13804 |
| 37   | Esterase D           | 31.5/7.0               | P10768           |
| 38   | Electron transfer flavoprotein α-subunit | 35.1/8.8 | P13804 |
| 39   | Δ3,5-Δ2,4-Dienoyl-CoA isomerase | 36.0/7.1 | Q13011 |
| 40   | Malate dehydrogenase, cyto | 36.3/7.5 | P40925 |
| 41   | Transaldolase        | 37.5/6.7               | P37837           |
| 42   | Alcohol dehydrogenase (NADP +) | 36.4/6.7 | P14550 |
| 43   | Unmatched             |                        |                  |
| 44   | Unmatched             |                        |                  |
| 45   | α-Enolase            | 47.0/7.5               | P06733           |
| 46   | Isocitrate dehydrogenase (NADP) cytoplasmic | 46.7/6.7 | O75874 |
| 47   | Elongation factor Tu, mitochondrial | 49.5/7.8 | P49411 |
| 48   | Isocitrate dehydrogenase (NAD) subunit | 37.6/6.9 | P50213 |
| 49   | Heterogeneous nuclear ribonucleoproteins C1/C2 | 33.3/5.1 | P07910 |
| 50   | Plectin 1 or its isoforms | 51.8/5.6 | Q15149 |
| 51   | Hypothetical protein KIA A0196 | 134/6.5 | Q12768 |
| 52   | Hypothetical protein KIA A0196 | 134/6.5 | Q12768 |
| 53   | Creatine kinase, B chain | 42.6/5.5 | P12277 |
| 54   | Anchor protein 350 kDa | 45.4/5.0 | Q99966 |
| 55   | Keratin type II      | 53.5/5.6               | P05787           |
| 56   | Probable protein disulfide isomerase ER | 56.8/5.6 | P30101 |
| 57   | T-complex protein 1, β subunit | 57.5/6.4 | P78371 |
| 58   | Succinyl-CoA-3-ketoacid coenzyme A transferase | 56.2/7.6 | P55809 |
| 59   | Unmatched             |                        |                  |
| 60   | D-3-phosphoglycerate dehydrogenase | 56.7/6.7 | O43175 |
expected from the calculated molecular mass and pI value (molecular mass 29.6 kDa; pI 8.5) and possibly suggests a proteolytic processing of the expressed protein. Indeed, most of the tryptic peptides found in spot 14 (Fig. 5b) were located within the C terminus portion of the protein (>residue 80). The sensitivity of this approach was found suitable to identify proteins of lower abundance such as that of spot 66 corresponding to a G-coupled protein receptor, a membrane component normally present in relatively low copy number in cellular extract (<50,000 copies per cell). Table I summarizes the protein identification obtained for excised spots shown in Fig. 4a. The proteins were identified by using the list of peptide masses or sequence tags against database from SwissProt.

### Identification of Target Analytes Using Immobilized Antibody and IMAC Beads on a Chip Device

The ability to pack separation media directly on the microfluidic device prompted us to investigate its potential for affinity selection of trace-level target peptides in complex biological extract. The application of immunoaffinity chromatography was first evaluated using an IgG antibody specific to a human proto-oncogene c-myc tag. In this particular example, the murine anti-c-myc IgG/Iκ antibody 9E10 recognizes the c-myc tag peptide epitope EQKLISEEDLN. This c-myc epitope is also fused to expressed proteins to facilitate their recovery from bacterial culture extracts. To evaluate this application, spiked c-myc solutions were spiked at concentration levels of 20–1000 ng/ml in a human serum. The biological fluid was first dialyzed against a 100-kDa cut-off membrane to remove components interfering with the immunoaffinity selection such as endogenous antibodies binding to free protein G sites. The pH level of the dialyzed serum (originally pH 7.9) was adjusted to 7 to enhance binding of the antigen. An aliquot of 50 μl of the spiked serum was loaded on the chip, and the immobilized antibody beads were subsequently rinsed with deionized water (10 μl) to remove unbound material. The selected antigen was released from the antibody beads using 10 μl of 0.1 M formic acid, and an electrokinetic injection between wells D and A (Fig. 1a) was applied to introduce the solution on the chip.

Fig. 6 shows the on-chip selection of a 20 ng/ml spike in human serum. The RIE for the expected c-myc immunogen is presented in Fig. 6a, together with the extracted mass spectrum for the peak detected at 0.9 min (Fig. 6b). The corresponding mass spectrum shows an abundant doubly charged ion at m/z 622.8 (a) and 687.3 (c) and extracted mass spectra for peaks identified at 0.90 (b) and 0.98 min (d). A total of 50 μl of a 100-kDa dialyzed human plasma sample was passed through the antibody beads, and the target peptides were released following a 1-μl wash with 0.2% HCOOH prior to injection on the chip-CE-nESMS device.

### Table I

| Spot | Protein or sequences                                      | Molecular mass (Da)/pI | Accession number |
|------|----------------------------------------------------------|------------------------|-----------------|
| 61   | Dihydrolipoamide dehydrogenase, mitochondrial            | 54.2/8.0               | P09622          |
| 62   | Glutamate dehydrogenase 1 precursor                     | 61.4/8.0               | P00367          |
| 63   | Phosphoenolpyruvate carboxykinase                        | 67.0/6.6               | Q16822          |
| 64   | Transformation-sensitive protein IEF SS                  | 62.6/6.8               | P31948          |
| 65   | Unmatched                                                |                        |                 |
| 66   | G protein-coupled receptor kinase GRK6                   | 66.0/8.3               | P43250          |
| 67   | Isoform of ADAM 17 precursor                             | 59.6/5.6               | P78536          |
| 68   | Mitochondrial stress-70 protein precursor                | 73.8/6.2               | P38646          |
| 69   | Heat shock cognate 71-kDa protein                        | 70.9/5.4               | P11142          |
| 70   | Unmatched                                                |                        |                 |
| 71   | Tubulin α-1 chain, brain-specific                        | 50.2/5.1               | P04687          |
| 72   | 78-kDa glucose-regulated protein precursor               | 72.1/5.1               | P11021          |
tive enrichment and preconcentration of target analytes was further investigated for the identification of phosphopeptides from in-gel protein digests. Such analyses have been very challenging in view of the low abundance of gel-isolated proteins, the expected recovery yields of tryptic peptides from in-gel digests, and the requirements for enhanced sequence coverage compared with that needed for simple protein identification. An earlier report from Roepstorff and co-workers (28) indicated that comprehensive phosphoproteome mapping experiments typically require low picomolar levels of gel-isolated proteins when using IMAC affinity selection. Preliminary investigations on IMAC separations were conducted using both Fe and Ga as chelating metals. A higher recovery yield was obtained on synthetic phosphopeptides using Fe instead of Ga, though better selectivity was achieved using Ga, consistent with previous studies (29).

An example of application of the present chip device for in-gel phosphoprotein digest is shown in Fig. 8 for the analysis of 2 pmol of α-casein (P02662) following one-dimensional SDS-PAGE separation. As indicated in Fig. 8, three phosphopeptides were separated on the IMAC chip-CE-nESMS system. For the purpose of comparison, the extracted mass spectrum corresponding to the original in-gel tryptic digest analyzed without IMAC selection is presented in Fig. 8a. In this case, the most abundant ions corresponded to non-phosphorylated peptides, whereas phosphopeptides (indicated by asterisks) are very weak. In contrast, the extracted mass spectrum obtained from the IMAC chip-CE-nESMS separa-
tion (Fig. 8b) of the same sample is dominated by distinct multiply charged phosphopeptide ions. The doubly protonated ions at \( m/z \) 976.96 and triply protonated ions at \( m/z \) 651.32 were assigned to the singly phosphorylated tryptic peptide T\(_{104-119}\) with a single skipped cleavage. The phosphopeptide ions observed at \( m/z \) 972.32 and 964.34 correspond to the oxidized and non-oxidized form of the doubly phosphorylated tryptic peptide T\(_{43-58}\). This oxidation is associated with the presence of the methionine residue on the peptide. A third phosphopeptide is observed in Fig. 8b at \( m/z \) 830.9 and was assigned to the tryptic fragment T\(_{106-119}\) from \( \alpha \)-casein. A peptide with a molecular mass of 1538.24 Da was observed in Fig. 8b; however this component could not be assigned to any of the expected tryptic fragments. The ability to conduct on-line IMAC and chip-CE-nESMS analysis is also illustrated from the reconstructed ion profiles of each of the phosphopeptide ions as indicated in Fig. 8c–e. The extracted mass spectrum for each peptide is also presented in Fig. 8f–h.

The sensitivity of the present system also enabled the characterization of the phosphorylation sites using on-line MS-MS. This is illustrated in Fig. 9 for a separate injection of a 2 pmol in-gel digest of \( \alpha \)-casein where the doubly charged precursors \( m/z \) 830.9 (T\(_{106-119}\)) and 964.4 (T\(_{43-58}\)) shown in Fig. 8 were selected for MS-MS analyses. In both cases, tandem mass spectrometry provided abundant y-type fragment ions from which the amino acid sequence could be deduced unambiguously. The MS-MS spectrum of \( m/z \) 830.9 (Fig. 9a) showed a single neutral loss of \( \text{H}_3\text{PO}_4 \) (\( m/z \) 732.4) consistent with the presence of a monophosphorylated residue. The location of this modification was assigned to Ser\(^{115}\) based on the observation of fragment ions at \( m/z \) 671.4 and 504.3 corresponding to the phosphorylated serine. Similarly, the MS-MS spectrum of \( m/z \) 964.4 (Fig. 9b) showed consecutive neutral losses of two of \( \text{H}_3\text{PO}_4 \) groups suggesting the presence of two phosphorylated residues. The location of the modification sites was based on the observation of fragment ions at \( m/z \) 1562.6 and 1248.6 suggesting that both Ser\(^{46}\) and Ser\(^{48}\) are phosphorylated.

**DISCUSSION**

In the present microfluidic system, a 96-well autosampler was integrated to a flow through channel on the chip and enabled the sequential introduction of protein digests and preconcentration of trace-level peptides on either adsorption or affinity selection media in a fully automated manner. A network of intersecting channels of dimensions significantly smaller than the flow through channel facilitated sample injection and separation on the chip without contamination of the fluids in the different manifolds. This chip design allowed the injection of 10 \( \mu \)l of tryptic digests with low fmol detection limits and a throughput of 12 samples/per h, with minimal carryover between consecutive injections. Desorption of tryptic peptides is typically achieved using 200 nl of organic buffer, which provided a sample utilization of \( \sim 5\% - 10\% \). Although this system enabled the analysis of \( \sim 200 \) fmol of in-gel protein digests as shown for both peptide standards and in-gel digests of human prostatic cell extracts, we antici-
ipate that this sensitivity may not be sufficient to address the more challenging proteomics applications requiring the detection of low protein expression (<5000 copies/cell). In this respect, the ideal microfluidics system must be capable of sampling a high proportion of the available protein digests even when present in denaturing agents. Such capabilities will require smaller sample desorption volume and precise control of the autosampler and the microfluidic manifold, and efforts are presently underway in our laboratory to incorporate these features on a modular microsystem.

The ability to manipulate larger volumes (>50 µl) of complex sample extracts and selectively enriching target analytes in a relatively short elution plug (typically <1 µl) is of significant analytical value to immunoaffinity separation. This was illustrated in the present study for the selection of 20 ng/ml of c-myc peptide in human plasma with a signal/noise ratio >10:1. Obviously, lower detection limits are expected for larger sample loading, and this could be of practical value when analyzing the binding of pharmaceuticals to receptor immobilized on affinity beads or for the enrichment of recombinant proteins from complex *Escherichia coli* extracts as shown in previous LC-MS applications from this laboratory (30).

The present investigation also demonstrated the application of IMAC beads for the selection of phosphopeptides from in-gel digests. Such studies require a higher level of sequence coverage compared with protein identification using either peptide mass fingerprinting or MS-MS microsequencing approaches, and low picomoles levels of tryptic digests are typically needed to obtain satisfactory results (28). Although we successfully demonstrated the integration of IMAC beads on microfluidic devices for the affinity capture of phosphopeptides from 2 pmol of in-gel digest of α-casein, the present approach might be of limited application in view of the relatively low abundance of naturally occurring phosphoproteins from cell extracts (typically <1000 copies/cell) and their variability in site occupancy. To address this sensitivity issue, we are currently evaluating alternative affinity strategies using β-elimination of phosphopeptides, followed by the addition of biotinylating reagent on the modified site (31).

The present microfluidic design offers a number of advantages in terms of ruggedness, reusability, and ease of integration of adsorption media for sample preconcentration, online digestion (29), or selective enrichment of target analytes through affinity beads. Although the present sampling device was applied to a small subset of human prostatic cell extract, it illustrates that the autosampler chip-CE device interfaced to a quadrupole/time-of-flight instrument device can play a pivotal role in accelerating the pace of protein identification in proteomics investigations. The capability of integrating modular units on the microfluidic device also brings us closer to a lab-on-a-chip system where the different steps involving sample injection, digestion, adsorption, and selective pre-concentration can be performed in a comprehensive fashion.

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Present address: Caprion Pharmaceuticals, 7150 Alexander Fleming, St-Laurent, Quebec H4S 2CB, Canada.

To whom correspondence should be addressed. Tel.: 613-998-0326; Fax: 613-941-1327; E-mail: pierre.thibault@nrc.ca.

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