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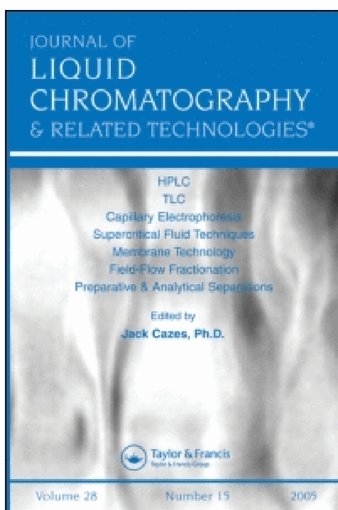

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Capillary-LC-μESI-MS/MS and Nano-LC-Nano ESI-MS/MS Analysis Using a Single Binary Pump Capillary LC System: Applications in Proteomics

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Capillary-LC-μESI-MS/MS and Nano-LC-Nano ESI-MS/MS Analysis Using a Single Binary Pump Capillary LC System: Applications in Proteomics

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Abstract: We describe the use of a single pump capillary-LC system (flow rate: 1–100 μL/min) to perform both capillary-LC-μESI-MS/MS and nano-LC-nano ESI-MS/MS (flow rate: 50–1000 nL/min) analysis and its applications in proteomics. A highly constant nanoflow (180 nL/min) delivery has been achieved by using a capillary binary-pump with electronic flow control and a flow splitter with compensation for the viscosity changes during gradient elution. A column switching technique was successfully used in nano-LC-nanoESI-MS/MS analysis to provide two flow paths, one for sample loading with a flow rate of 15 μL/min directly from the capillary pump, the other for nanoflow gradient elution with a flow rate of

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180 nL/min after flow splitting. In comparison with the capillary-LC-μESI-MS/MS at a flow rate of 3 μL/min, this nano-LC-nano ESI-MS/MS analysis improved sensitivity by more than 50 times. It has been successfully used to identify protein spots from 2DGE, as well as protein bands from 1DGE, after in-gel tryptic digestion. Similar to any commercial nano-LC system, this novel nano-LC configuration can be used to perform MudPIT analysis, as well as offline capillary-SCX/nano-RPLC-nanoESI-MS/MS analysis. Our data has shown the unnecessary need for either a second pump or an expensive nano-pump to do nano-LC analysis as most commercial nano-LC system requires, and the increased sensitivity, functionality, and flexibility of a simple capillary-LC system, which is very useful to typical analytical laboratories for multiple-purpose usages of limited resources.

Keywords: Capillary-LC-μESI-MS/MS, Nano-LC-nano ESI-MS/MS, Column switching, Two-dimensional liquid chromatography, Proteomics, Human plasma

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INTRODUCTION

Proteomics research enables us to investigate systems-level biology by allowing global profiling of proteome of cells, tissues, and body fluids, and providing means to characterize their changes during physiological and pathophysiological conditions. The ability of mass spectrometry to identify minute amounts of proteins from complex mixtures is a primary driving force in proteomics.[1] Initial proteomics efforts relied on protein separation by two-dimensional gel electrophoresis (2DGE) and subsequent protein spot identification by mass spectrometry after in-gel digestion.[2] Protein spot identification is achieved by peptide mass fingerprinting (PMF) using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOFMS).[3] More reliable protein spot identification can be achieved by partially sequencing several peptides from the digest of the protein spot, using nano-electrospray tandem mass spectrometry (nanoESI-MS/MS).[4] While MALDI-TOFMS is normally used to analyze relatively simple peptide mixtures, nanoESI-MS/MS can be used to analyze complex peptide mixtures, as it can be easily coupled to liquid-based separation tools.

Methods for increasing detection sensitivity are of great interest in proteomics because the dynamic range of protein expression in human cells can be 10^5[5] and many proteins of interest are expressed in low abundance. It has previously been shown, that the use of smaller ESI emitters with tip dimensions in the range of 1–25 μm and flow rates in the low-nanoliter per minute (nanoESI) greatly lower the MS detection limit into sub-fmol range.[6,7] In order to achieve high sensitivity, the nanoESI-MS/MS method requires desalting to remove buffer salts from the sample and pre-concentration steps.[6] Recently, we reported a novel multiplexed solid-phase-extraction system directly linked to a nanoESI source for high throughput (2.5 min/spot)

2DGE spot identification by using a flow rate of 500 nL/min.[8] More sensitive, but lower throughput analysis can be achieved by using nanoflow (10–1000 nL/min) reversed-phase liquid chromatography (nano-RPLC) coupled with the nanoESI source.[9–13] Since the nano-RPLC not only desalts and pre-concentrates samples but also separates peptides, this approach is suitable for the analysis of peptide mixtures that are more complex than 2DGE spot digests, e.g., 1DGE band[11] and cell lysate digests.[12,13] Although high-efficiency nano-RPLC with a peak capacity as high as 1,000 has been reported recently,[12,13] even higher peak capacity is still in demand for the separation of extremely complex peptide mixtures, such as total cell lysate digests with hundreds of thousands peptides. A micro-preparation method to fractionate highly complex peptide mixtures before nano-RPLC-nano ESI/MS/MS analysis, using diverse separation schemes other than reversed-phase separation, have been explored and the most popular at present is strong cation exchange (SCX).[14–18]

Today, at the heart of proteomics is the use of highly-efficient methods to separate protein/peptide mixtures prior to analysis by mass spectrometry. Multidimensional separation such as 2DGE and multidimensional chromatography are being utilized for the high-efficiency separation, and nano-RPLC plays an important role in both gel- and non-gel- based methods. Nano-LC systems, which are also capable of multidimensional chromatographic separations, are commercially available. They include Agilent 1100 nanoflow proteomics solution, LC Packings Ultimate™ Plus, Waters nanoACQUITY UPLC, Micro-Tech X'TremeSimple™, to name a few. Because nano-LC requires the use of low μL/min flow rate to load low μL sample volume and middle-nL/min flow rate for separation, commercial nano-LC systems usually employ dual pumps: a capillary pump for sample loading and a nano-pump for gradient elution. In contrast, capillary-LC is simpler, cheaper, and more robust, while providing similar high efficient separation.[13] Consequently, it is also widely applied in the proteomics study.[19–22] In fact, typical analytical laboratories are usually equipped with a single pump conventional (flow rate 100–1000 μL/min) and/or capillary (flow rate 1–100 μL/min) -LC system for multi-purpose use rather than a nano-LC system that is dedicated solely to proteomic study.

Here, we describe a novel design to economically convert a capillary-LC system with a capillary binary-pump into an easy-to-use nano-LC system for a proteomics study. As configured, this system increases sensitivity, functionality, and flexibility of a simple capillary-LC system. We demonstrate that at least a 50-fold increase in sensitivity can be achieved by performing nano-LC-nano ESI-MS/MS instead of capillary-LC-μESI-MS/MS. Similar to commercially available nano-LC systems; this novel configuration also has the flexibility to perform two-dimensional chromatography (SCX/RPLC). It has been successfully used for 2DGE spot identification and 1DGE band analysis.

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EXPERIMENTAL

Materials and Chemicals

Formic acid, deionized water, and acetonitrile were purchased from VWR international, Inc. (West Chester, PA). Myoglobin from horse heart was purchased from Sigma Chemical Co. (St. Louis, MO). Sequence grade trypsin was purchased from Promega (Madison, WI, USA). MassPREP™ protein digestion standards (enolase, phosphorylase b, bovine hemoglobin, alcohol dehydrogenase, and bovine serum albumin) were purchased from Waters (Milford, MA, USA).

Capillary-LC and Nano-LC

An Agilent (Palo Alto, CA, USA) 1100 series capillary-LC system consisting of the following components was used: solvent cabinet, vacuum degasser, capillary binary-pump with two flow sensors (1–20 μL/min and 10–100 μL/min), micro well-plate autosampler with two injection loop (8 and 40 μL), and micro 10 port/2 position switching valve box with holder (model G1163A). The capillary-LC configuration is illustrated in Figure 1 with a 300 μm i.d. × 150 mm PepMap™ C18 column (100 angstrom, 3 μm particle) from LC Packings (Sunnyvale, CA, USA). The nano-LC configuration is illustrated in Figure 2 with an Acurate™ 100 flow splitter, capable of delivering a precise flow rate of as low as 50 nL/min at an input flow rate of 50–100 μL/min, a 300 μm i.d. × 5 mm PepMap™ C18 trap column (100 angstrom, 5 μm particle), and a 75 μm i.d. × 150 mm PepMap™ C18 column (100 angstrom, 3 μm particle) from LC Packings (Sunnyvale, CA, USA). More detailed information on the operation of the capillary-LC and nano-LC is described in Results and Discussion section.

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μESI-MS/MS and nano ESI-/MS/MS

The μESI-MS/MS measurement was performed by using a Bruker Daltonic (Billerca, MA, USA) Esquire quadrupole ion trap mass spectrometer with an API (atmosphere pressure ionization)–electrospray interface capable of operating at flow rates from 1 to 1000 μL/min. During peptide sequencing analysis, an automatic MS to MS/MS switching feature was used with standard mass range (m/z 50–2,200) and unit resolution. The MS and MS/MS scans were set at the same mass range of m/z 200 to 2, 200. Two or three ions from m/z 500 to 1,100, above a preset intensity threshold of 20,000, were simultaneously selected during the MS scan for subsequent MS/MS experiments. Each scan took approximately 3 s and was acquired with an maximum accumulation time of 60 ms, ICC (Ion Charge Control)

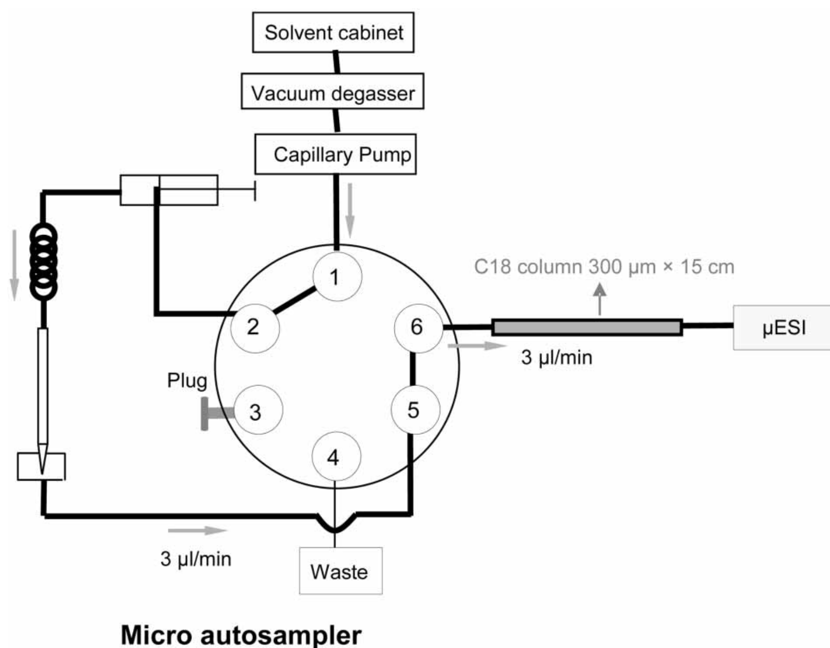

Figure 1. Schematic representation of the capillary-LC-μESI-MS/MS system showing its configuration.

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target of 50,000, and 7 spectra in average. The nanoESI-MS/MS analysis was performed by using a New Objective (Woburn, MA, USA) PicoView 200 nanoESI source capable of operating at flow rates from 1 to 1000 nL/min. During peptide sequencing analysis, mass spectrometric parameters used in μESI-MS/MS analysis were also employed.

All MS/MS data were analyzed using the Mascot search engine (http://www.matrixscience.com, Matrix Science Ltd., London, U.K.) against the NCBInr database. The database searches were performed with fixed modification of cysteine residue (carbamidomethylation, +57 Da) and variable modification of methionine residue (oxidation, +16 Da). Peptide mass tolerance and fragment mass tolerance were set at ±2.0 Da and ±0.8 Da, respectively.

Human Serum/Plasma Protein Depletion

The high abundant proteins in serum/plasma were depleted by employing an Agilent (Palo Alto, CA, USA) multiple affinity column using the protocol recommended by the vender.

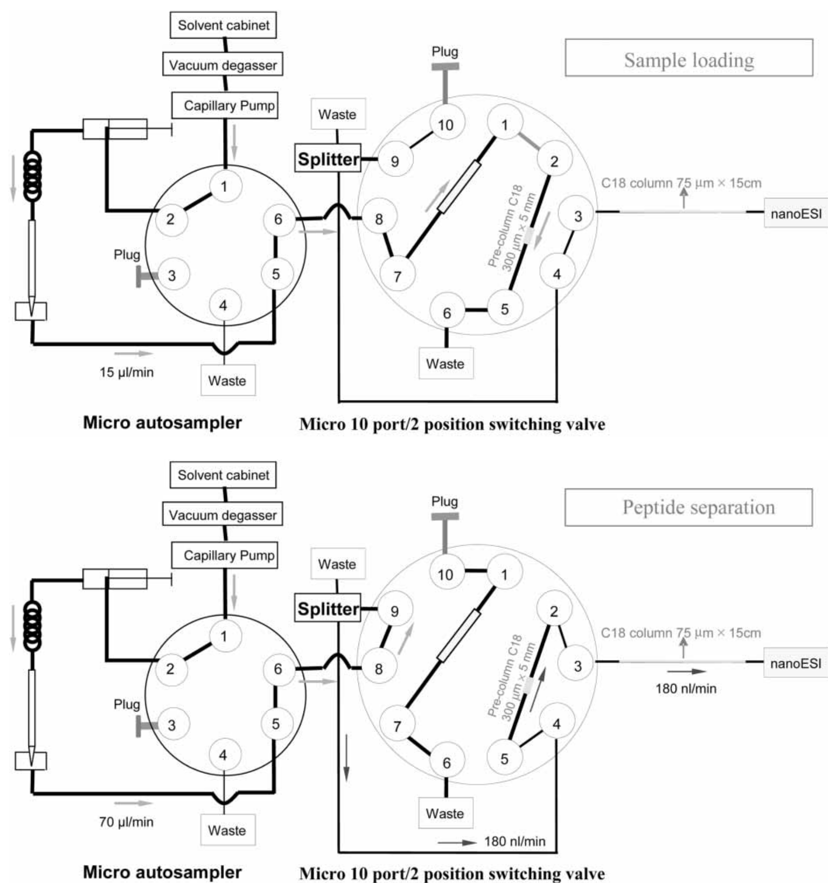

Figure 2. Schematic representation of the nano-LC-nanoESI-MS/MS system showing its construction with only one capillary pump in combination with a micro 10 port/2 position switching valve and a splitter. The C18 trap and separation columns are labelled. The optional SCX column for MudPIT analysis is shown connected between ports 1 and 7. There are two system working positions, where the switching valve changes its position for sample loading (A) and peptide sequencing (B).

ZIGE and In-Gel Digestion

2DGE and in-gel digestion was performed by using a previously reported procedure.[23]

RESULTS AND DISCUSSION

A conventional-LC and a capillary-LC-ESI/MS/MS system have been used in our lab for multiple purposes such as clinical analysis,[24] carbohydrate

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characterization,[25] DNA damage measurement,[26] DNA methylation determination,[27] and proteomics research.[28] According to our experience, capillary-LC with a flow rate of 1–100 μL/min offers an advantage for the analysis of samples with limited sample amount and/or concentration, while offering the same robustness as conventional-LC with a flow rate of 100–1000 μL/min. As previously documented, due to reduced chromatographic dilution, miniaturized LC enhances detection sensitivity when it employs concentration-sensitive detection devices.[29,30] With regard to ESI/MS detection, Banks[31] demonstrated that LC/MS responses can be increased as much as 163-fold by decreasing the LC flow rate from 1000 to 1.8 μL/min and also by reducing the column diameter from 4.6 to 0.25 mm. In our proteomics studies, capillary-LC separations have been performed at a flow rate of 3 μL/min with a 300 μm × 150 mm C_{18} column with the configuration as shown in Figure 1. The particular LC scale was selected to achieve the robustness of the operation, as well as to meet the requirement of high sensitivity. As previously reported by Abian et al.,[30] the loading capacity of a 300 μm × 150 mm C_{18} column is approximately 485 nL, and a few μL sample volume can be loaded only in a solvent with low eluotropic strength, so that it can be pre-concentrated at the top of the column. In our experiment, an 8-μL sample volume was usually injected at a flow rate of 3 μL/min so that sample loading could not cause significant delay of the analysis.

The gradient elution program used for our capillary-LC separation is as follows: 0 min, 5% B; 2 min, 5% B; 30 min, 50% B; 36 min, 100% B; 38 min, 100% B; 40 min, 5% B; and 60 min, 5%B (solvent: A = H_2O + 0.1% formic acid; B = ACN + 0.1% formic acid). To test the proteomics capability of the capillary-LC-μESI-MS/MS system, tryptic digestion of myoglobin solution (5.9 pmol/μL) was used following serial dilution. Our results demonstrated that 1 pmol myoglobin tryptic digest can be successfully identified with a probability based Mowse score higher than 200. Four peptides: LFTGH PETLE K (m/z 636.6), VEADI AGHGQ EVLIR (m/z 803.9), HTTVV LTALG GILK (m/z 689.9), and HPGDF GADAQ GAMTK (m/z 751.8) were generally identified. Figure 3 shows the selected ion chromatogram of these peptides. Therefore, the analysis of 1 pmol myoglobin tryptic digest was employed as a standard quality control operation procedure in our capillary-LC-μESI-MS/MS application for proteomics study. The capillary-LC-μESI-MS/MS analysis has been successful to identify proteins excised from coomassie-stained 2DGE spot and 1DGE band with relatively strong intensity (detailed data not shown).

At least 3 factors contribute to sensitivity in capillary-LC-μESI-MS/MS sensitivity: capillary-LC, μESI, and mass spectrometric measurements. In order to improve sensitivity, we then focused our study on using nano-LC and nanoESI. A novel nano-LC-nanoESI-MS/MS configuration was developed by using the single binary-pump capillary-LC system, and

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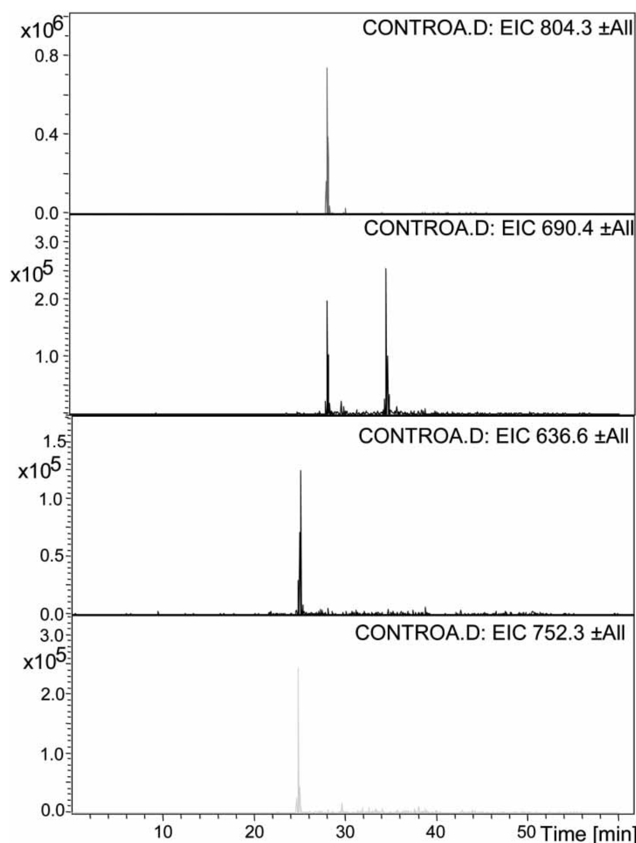

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Figure 3. Selected ion chromatograms of peptides from 1 pmol myoblobin tryptic digest detected by capillary-LC-μESI-MS/MS. The following gradient was used for the capillary-LC separation: 0 min, 5% B; 2 min, 5% B; 30 min, 50% B; 36 min, 100% B; 38 min, 100% B; 40 min, 5% B; and 60 min, 5%B (solvent: $A = H_2O + 0.1\%$ formic acid; $B = ACN + 0.1\%$ formic acid).

Figure 2 shows its configuration. Two additional devices, an Acurate™ 100 flow splitter from LC Packings and a micro 10 port/2 position switching valve box with holder from Agilent were also employed. The Acurate™ 100 flow splitter[29] is capable of delivering a precise flow rate of as low as 50 nL/min at an input flow rate of 50–100 μL/min. It is compatible with any type of solvent delivery system including reciprocating pumps and syringe pumps. It also compensates for the viscosity changes during gradient elution, and hence, results in highly constant flow delivery.[29] The split ratio was defined by connecting pre-adjusted calibrators to the splitter, similar to that used in LC Packings Ultimate™ Plus nano-LC system (http://www.lcpackings.com/products/Instruments/UltiMatePlus02.htm).

A problem with using a splitter to deliver nanoflow rate is that there is always the potential for unpredictable changes in flow rates caused by unexpected backpressure changes or clogging. For this reason, splitless nano-LC systems such as Micro-Tech X'TremeSimple™ (http://www.microlc.com/Html/Products/Systems/TremeSimple Family.htm) and Waters nanoACQUITY UPLC (http://www.waters.com/Waters Division/Contentd.asp?ref = PSTD-5ZBBSC) are appearing in the marketplace. Alternatively, flow splitting nano-LC system such as Agilent 1100 nanoflow proteomics solution (http://www.chem.agilent.com/Scripts/PDS.asp?lPage = 8473) employs an electronic flow control that measures and controls the flow rate. This assures the flow stability throughout the analysis, even with unexpected changes in the backpressure. In our configuration, the Agilent 1100 capillary binary-pump had such a function and, therefore, a constant input flow rate to the splitter was guaranteed. The splitter contained a replaceable inline nanofilter at the inlet to trap particles carried by the mobile phase. An additional low dead volume (10 nL) inline nanofilter (Upchurch Scientific, Oak Harbor, WA, USA) was placed behind the flow splitter calibrator for additional protection of the system from clogging. For over eight months of operations we monitored the flow rate and reached a conclusion that a highly constant flow was reproducibly delivered as expected. In our application, a 75 μm × 150 mm C_{18} column was selected for the nano-LC separation at a flow rate of 180 nL/min. An input flow rate of 70 μL/min was determined and utilized to generate an output flow rate of 180 nL/min from the splitter. A gradient delay time of 10 min from the flow splitter was also determined and taken into consideration to program the gradient elution.

Since the loading capacity of a 75 μm × 150 mm C_{18} column is less than 3 nL,[29] a column switching technique has previously been developed to load low μL sample volumes for nano-LC separation with the assistance of a trap column.[30] Because this technique requires a low μL/min flow rate for sample loading and a nL/min flow rate for separation, commercial nano-LC systems usually employ dual pumps, a capillary pump for sample loading and a nano-pump for gradient elution. In our configuration (Figure 2), a capillary pump was incorporated with a 2 position/10 port automatic switching valve to which a flow splitter, a 300 μm i.d. × 5 mm C_{18} trap column, and a 75 μm i.d. × 150 mm C_{18} column were connected. During injection at a flow rate of 15 μL/min (Figure 2A), the flow from the capillary pump passed into the trap column, but bypassed the flow splitter and the separation column, and was directed to waste. This design allowed the washing-off of salts and other contaminants that would be problematic for MS analysis. During separation at a flow rate of 70 μL/min from the capillary pump (Figure 2B), the flow passed through the flow splitter and reduced to 180 nL/min, then passed through the trap column, the separation column, and the nanoESI source, sequentially. In order to maintain a highly efficiency nano-LC separation and to avoid peak dispersion, a low dead

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volume switching valve (stator 20°, 27.2 nL/port; stator 45°, 30.5 nL/port; rotor seal, 25.0 nL/groove) and 20 μm i.d. fused silica capillary for the connection of the columns to the switching valve were used, respectively.

The LC flow programs are shown in Table 1. Protocol #1 was very successful for the identification of 20 fmol myoglobin tryptic digest with a probability based Mowse score higher than 200. Figure 4 shows the selected ion chromatogram of m/z 636.6, 804.4, 690.3, and 752.3 ± 0.5 Da. Comparing Figure 4 (20 fmol myoglobin digest) with Figure 3 (1 pmol myoglobin digest), we concluded that the nano-LC-nanoESI-MS/MS improved the

Table 1. Nano-LC flow programs with a single binary-pump capillary-LC system using the novel configuration shown in Figure 2

Time (min)						
Protocol #1	Protocol #2	Valve position	Flow rate (μL/min)	Buffer A[a] (%)	Buffer B[b] (%)	Function
0.00	0.00	A	15	98	2	Sample loading starts
5.45	5.45	B	15	98	2	Valve switches
5.50	5.50	B	15	98	2	Flow adjusts
6.50	6.50	B	70	98	2	Gradient elution starts
8.50	9.50	B	70	85	15	Gradient changes
30.50	69.50	B	70	55	45	Gradient changes again
33.00	73.00	B	70	10	90	Column cleaning starts
35.50	75.50	B	70	10	90	Column cleaning ends
37.50	77.50	B	70	98	2	Gradient delay compensates; column equilibrates
77.50	117.50	B	70	98	2	Column equilibration ends
78.50	118.50	B	15	98	2	Flow adjusts
78.55	118.55	A	15	98	2	Valve switches
80.00	120.00	A	15	98	2	Ready for sample loading again

[a]Buffer A: 0.1% aqueous formic acid.
[b]Buffer B: 0.1% formic acid/acetonitrile.

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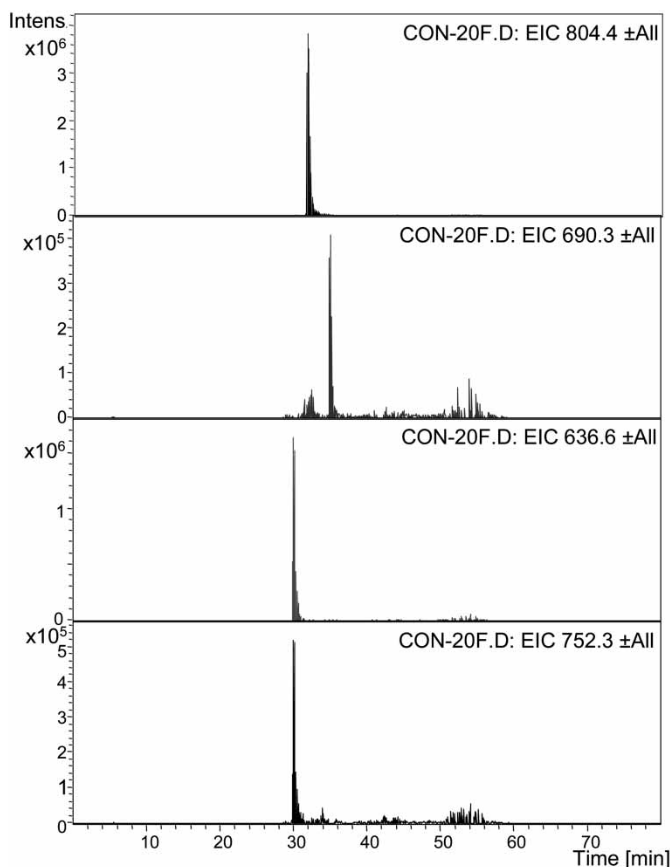

Figure 4. Selected ion chromatograms of peptides from 20 fmol myoblobin tryptic digest detected by nano-LC-nanoESI-MS/MS. The nano-LC flow program is shown in Table 1 and protocol #1 is used.

sensitivity more than 50 times over the capillary-LC-μESI-MS/MS analysis, since the selected ion currents were similar, even though the amounts of myoglobin digest analyzed were 50-fold different.

As in a commercially available nano-LC system, our nano-LC configuration can also be modified to do MudPIT analysis[17] by adding a SCX column between ports 7 and 1 of the switching valve depicted in Figure 2. The operation of the MudPIT analysis will essentially be the same as the nano-LC-nano ESI-MS/MS in this added configuration. During sample injection, the SCX column will be inline with the trap column and the flow path is the same as in Figure 2A. Most peptides will be retained in the SCX column and the flow-through peptides will be trapped in the trap column. The flow-through of the trap column, which is mostly salts and other contaminants, is

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directed to the waste. The trap column can then be transferred into the nanoflow path (Figure 2B). Increasing concentration of organic solvent would elute the peptides that are concentrated on the top of the trap column, and a further separation will be achieved on the nano-column. The peptides, which are retained in the SCX column, can be eluted stepwise by injections of 20 μL salt solutions with increasing concentrations from the autosampler and trapped in the trap column. After each salt injection, the trap column will be transferred into the nano-flow path, as shown in Figure 2B, and the peptides eluted and separated with increasing concentration of organic solvent.

The nano-LC system from Agilent (so called Nanoflow Proteomics Solution) has been described recently by Nagele et al.[15] In this system, by using dual pumps and dual trap columns, sample loading was accomplished during nano-LC elution. Therefore, the analysis time for each sample was equal to the nano-LC elution time. In our configuration, due to the use of only one pump, sample loading and nano-LC elution were performed, sequentially. Therefore, the analysis time for each sample was equal to the sample loading time plus the nano-LC elution time. However, in order to achieve high peak capacity, nano-LC elution usually takes at least 1 hour (protocol #1). When the peptide mixture is very complex, even longer nano-LC elution times, such as 100 min,[15] 2 hours (protocol #2), or more can be used. In contrast, sample loading usually takes only a few minutes. Thus, sample-loading time does not play a key role in the total analysis time, so that a dedicated capillary pump is absolutely required to perform this function. It can be potentially important, however, for high-throughput analysis of simple peptide mixtures. A solution for this, is by multiplying the flow path and utilizing isocratic elution with cheaper syringe pumps as we suggested recently.[8] A throughput of 2.5 min/sample has been achieved with this approach.

To demonstrate the separation of a complex peptide mixture, a mixture of the tryptic digestion of five standard proteins (enolase, phosphorylase b, bovine hemoglobin, alcohol dehydrogenase, and bovine serum albumin) were separated by using our novel nano-LC-nano ESI-MS/MS configuration with an 1 hour slow gradient and 2 hours total analysis time (protocol #2). As shown in Table 2, all proteins in the mixture were successfully identified with a high probability based Mowse score. Bovine hemoglobin was identified with both beta-A chain and alpha chain. A representative MS/MS spectrum of a peptide from enolase with m/z 878.0 is shown in Figure 5. The Mascot search easily interpreted this spectrum to be TAGIQIVADDLTVTNPK with some significant y ions and b ions, as shown in Figure 5. Due to its excellent separation capability of a complex peptide mixture, we propose that protocol #2 would be exploited on MudPIT analysis. Recently, offline capillary-SCX/nano-RPLC-nano ESI-MS/MS[16,18] has been demonstrated to be superior to MudPIT in proteomics applications. Our single pump capillary-LC system can be easily adopted to carry out offline capillary-SCX/nano-RPLC-nano ESI-MS/MS as follows. The first capillary-SCX

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Table 2. Mascot search results for the nano-LC-nano ESI-MS/MS analysis (protocol #2) of a complex peptides mixture from the tryptic digests of five standard proteins (100 fmol injection for each protein)

Protein	Mowse score	SC (%)	Peptides	Peptide sequence	Peptide mass	Peptide position
Enolase	406	19	7	SVYDSRGNPTVEVELTTEK	2123.54	10–28
				GNPTVEVELTTEK	1415.70	16–28
				NVNDVIAPAFVK	1286.32	68–79
				AVDDFLISLDGTANK	1577.36	89–103
				IGSEVYHNLK	1159.21	186–195
				TAGIQIVADDLTVTNPK	1753.84	313–329
				VNQIGTLSESIK	1288.64	347–358
Phosphrylase b	356	11	11	GLAGVENVTELK	1229.73	5–16
				GLAGVENVTELKK	1356.97	5–17
				DYYFALAHTVR	1354.78	38–48
				YEFGIFNQK	1145.55	149–157
				VLYPNDNFFEGK	1441.66	256–267
				TNFDAFPDK	1053.25	324–332
				VAAAFPGDVDR	1117.52	383–393
				TIFK	505.64	467–470
				VHINPNSLFDVQVK	1607.98	524–537
				VFADYEEYVK	1262.47	742–751
Bovine serum albumin	342	20	11	LVNELTEFAK	1164.55	66–75
				SLHTLFGDELCK	1417.99	89–100
				VATLR	558.76	101–105

(*continued*)

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Table 2. Continued

Protein	Mowse score	SC (%)	Peptides	Peptide sequence	Peptide mass	Peptide position
				ETYGDMADCCEK	1477.22	106–197
				YNGVFQECCQAEDK	1745.89	184–197
				YICDNQDTISSK	1443.63	286–297
				EYEATLEECCAK	1501.39	375–386
				HLVDEPQNLIK	1305.00	402–412
				CCTESLVNR	1138.10	499–507
				TVMENFVAFVDK	1398.61	569–580
				CCAADDKEACFAVEGPK	1927.52	581–597
Alcohol dehydrogenase	491	23	6	SIGGEVFIDFTK	1311.93	213–224
				YVRANGTTVLVGMPAGAK	1803.4	259–276
				ANGTTVLVGMPAGAK	1401.69	262–276
				CCSDVFNQVVK	1355.13	277–287
				SISIVGSYVGNR	1251.16	288–299
				VVGLSTLPEIYEK	1447.11	320–332
Bovine hemoglobin beta–A chain	167	32	4	LLVVYPWTQR	1274.40	30–39
				GTFAALSELHCDK	1447.73	82–94
				EFTPVLQADFQK	1421.04	120–131
				VVAGVANALAHR	1177.55	132–143
Bovine hemoglobin alpha chain	151	19	2	VGGHAAEYGAEALER	1528.33	17–31
				FLANVSTVLTSK	1277.99	128–139

Shown are probability based Mowse scores, sequence coverage (SC), number of identified peptides, their sequences and masses.

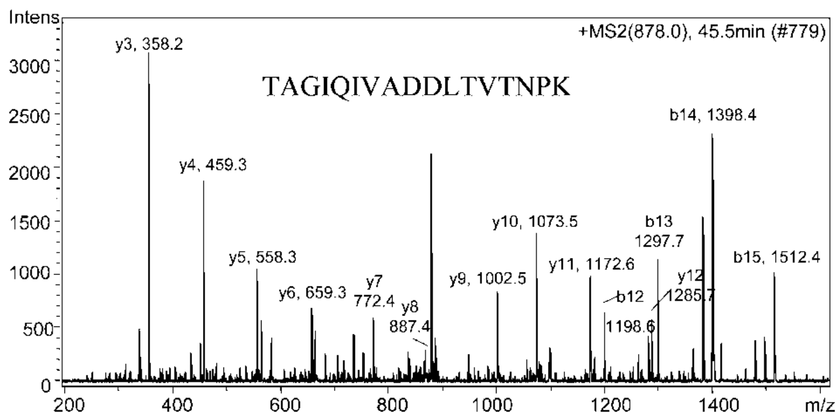

Figure 5. A representative MS/MS spectrum of a peptide from enolase with *m/z* 878.0, which was separated from a complex peptides mixture of the tryptic digests of five standard proteins (enolase, phosphrylase b, bovine hemoglobin, alcohol dehydrogenase, and bovine serum albumin) by nano-LC-nanoESI-MS/MS with protocol #2 (100 fmol injection for each protein).

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(320 μm i.d. × 10 mm) dimension can be performed by directly using the system with an additional microfraction collector at a flow rate of 10 μL/min. The second nano-LC-nano ESI-MS/MS dimension can be performed by using the configuration described above with protocol #2.

We have applied our nano-LC-nano ESI-MS/MS system to identify 2DGE spot and 1DGE band in a few proteomic studies (detailed data will be published elsewhere). Protocols #1 and #2 were used, respectively, for these analyses. The analysis of 20 fmol myoglobin tryptic digest using protocol #1 was used as a standard quality control operation procedure. Our results showed that very weak 2DGE spots visualized by CyproRuby fluorostaining (1–2 ng/spot sensitivity)[32] have been identified. Figure 6 shows an example of the application of the nano-LC-nano ESI-MS/MS system to identify 2DGE spots from human serum. High abundant proteins were depleted by the Agilent multiple affinity columns. More data on human serum/plasma proteome analysis is published in another paper.[33] Further application in MudPIT[17] and offline capillary-SCX/nano-RPLC-nanoESI-MS/MS[16,18] analyses are being tested with human serum/plasma samples after high abundant protein depletion with the Agilent multiple affinity column.

CONCLUSIONS

The novel configuration for nano-LC-nanoESI-MS/MS described here includes the use of a single capillary binary-pump, a flow splitter, an

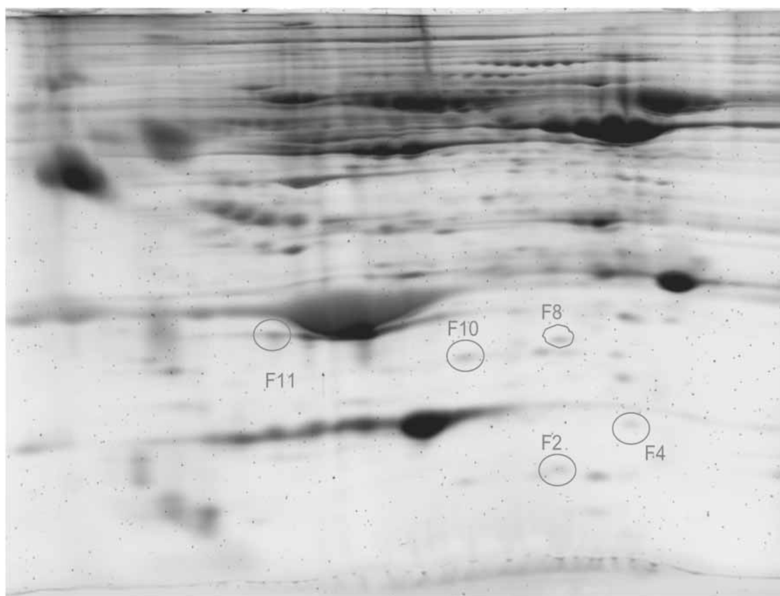

Figure 6. An example of the application of the nano-LC-nanoESI-MS/MS system for the identification of 2DGE spots from human serum after the removal of high abundant proteins by Agilent multiple affinity columns. F2, tropomyosin isoform; F4, glutathione S-transferase; F8, fibrin alpha C term fragment; F10, haptoglobin; F11, retinol-binding protein.

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automatic switching valve, and a nanoESI source. The data shows that the sensitivity obtained by nano-LC-nano ESI-MS/MS using this single pump capillary-LC system can be up to 50 fold higher than that obtained by capillary-LC-μESI-MS/MS using the same pump. Even more efficient separation, such as online capillary-SCX/nano-RPLC required by MudPIT and offline capillary-SCX/nano-RPLC can be also performed with the same configuration. Moreover, high-efficiency online and offline capillary-SCX/capillary-RPLC separation can also be achieved without the use of the flow splitter, as in the nano-LC configuration (Figure 2). Therefore, this configuration has increased not only the sensitivity, but also the functionality and flexibility of a simple single pump capillary-LC system. Of particular importance, is that this system is very economical due to the use of existing hardware (i.e., capillary-LC system and mass spectrometer) and the unnecessary need for either a second pump or an expensive nano-pump to do nano-LC analysis. The conversion of the systems is extremely simple and can be done in a few minutes.

While both capillary-LC-μESI-MS/MS and nano-LC-nano ESI-MS/MS offer comparable high-efficiency separation for proteomics research, the

former has the advantage of robustness and the later the advantage of sensitivity. For high throughput analysis of simple peptide mixtures, capillary-LC has the advantage of a much shorter gradient delay time; therefore a higher throughput can be achieved when a faster elution gradient is used. The use of a single pump capillary-LC system to perform nano-LC separation with the aid of our configuration, allows a single instrument, a capillary-LC system, to be applied to the two highly efficient separation techniques. In reality, the selection between capillary-LC-μESI-MS/MS versus nano-LC-nano ESI-MS/MS should depend on the complexity of the sample, the available sample amount, the sensitivity and the throughput requirements of the analysis, and the available sensitivity of the mass spectrometer.

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