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More Sensitive and Quantitative Proteomic Measurements Using Very Low Flow Rate Porous Silica Monolithic LC Columns with Electrospray Ionization-Mass Spectrometry

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The sensitivity of proteomics measurements using liquid chromatography (LC) separations interfaced with electrospray ionization-mass spectrometry (ESI-MS) improves approximately inversely with liquid flow rate (for the columns having the same separation efficiency, linear velocity, and porosity), making attractive the use of smaller inner diameter LC columns. We report the development and initial application of 10 μm i.d. silica-based monolithic LC columns providing more sensitive proteomics measurements. A 50-μm-i.d. micro solid-phase extraction precolumn was used for ease of sample injection and cleanup prior to the reversed-phase LC separation, enabling the sample volume loading speed to be increased by ~50-fold. Greater than 10-fold improvement in sensitivity was obtained compared to analyses using more conventional capillary LC, enabling e.g. the identification of >5000 different peptides by MS/MS from 100-ng of a *Shewanella oneidensis* tryptic digest using an ion trap MS. The low nL/min LC flow rates provide more uniform responses for different peptides, and provided improved quantitative measurements compared to conventional separation systems without the use of internal standards or isotopic labeling. The improved sensitivity allowed LC-MS measurements of immunopurified protein phosphatase 5 that were in good agreement with quantitative Western blot analyses.

Keywords: quantitative proteomics • ion suppression • label free approach • microSPE-nanoLC-ESI-MS • silica-based monolithic column • Western blot analysis

Introduction

Quantitative analyses of the array of proteins in cells or tissue (i.e., proteomics) enable applications that include fundamental studies of protein function(s), discovery of candidate disease biomarkers, and broad systems biology approaches.[1] Various combinations of separations with MS are now widely applied for proteomics measurements. The value of proteomics measurements increases with the quality of the separations used, the sensitivity and dynamic range of the MS instrument, and the quantitative utility of the data (e.g., the linearity of MS response with protein abundance).

Two general approaches for quantitative proteomics have been reported based on the coupling of liquid chromatography (LC) with ESI-MS.[2,3] One approach[4,5] uses stable isotope labeled versions of protein or peptide as effective internal standards for either relative quantitation (e.g., using a reference proteome) or absolute quantitation (i.e., when the abundances of an added species are known), and assumes relatively uniform ESI-MS responses for light- vs heavy-labeled versions of peptides or proteins. However, the isotopic labeling process can be incomplete, result in artifacts (e.g., due to reaction byproducts), increase sample complexity, may be impractical for applications involving large numbers of analyses,[6] and cannot directly provide information on the relative abundances of *different* species. The alternative "label-free" approaches for quantitative proteomics are based upon the comparison of peak intensities for the same species in different analyses,[6−8] often after normalization (e.g., by assuming constant relative abundances of "house-keeping" proteins between samples) to ideally account for variations in sample quantity or instrument performance between analyses.[8] However, in addition to differences in ionization efficiencies between different species, label-free quantitation of peptides using LC-ESI-MS commonly suffer from ionization suppression and matrix effects in ESI that cause nonlinear responses with abundance, as well as strongly biased detection efficiencies for different species.[9−16] These deleterious effects arise due to the combined effects of charge competition,[12,13,16−18] physical and chemical separation during the asymmetric charged fission processes of electro-

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sprayed droplets,[19−25] causing large fractions of analytes in higher flow rate separations to be converted to unanalyzed "charged residue" particles or large clusters vs measurable ions.[9,13,17,26] Analyses using the smallest bore capillary LC columns commercially available (operated at a linear velocity where the separation quality is optimal; ~300 nL/min from 75 micron i.d. capillaries) generally incur significant compound-to-compound variations and nonlinear ESI-MS responses with concentration, due at least in part to such factors. Therefore, differences in LC-MS peak intensities often do not correctly reflect changes in abundance.

It has been recognized that there are substantial advantages for ESI-MS analyses at very low liquid flow rates. ESI−MS analyses with flow rates of ~20 nL/min have been shown to significantly increase sensitivity compared to the higher flow rates that apply with LC separations.[27−30] The highly charged smaller initial droplets initially formed by such electrosprays (estimated of <200 nm diameter) more rapidly evaporate and efficiently produce the ions,[10,26] resulting in improved sensitivity or reduced sample consumption. As analyte ionization efficiencies are increased, detection biases also decrease as undesired matrix effects or ionization suppression effects are reduced or eliminated.[26,31,32] Overall efficiency for conversion of analyte ions from solution to gas phase ions has been predicted to approach 100% when both the liquid flow rate and analyte concentration are sufficiently low,[9,31,32] and it is well demonstrated that smaller i.d. columns at lower flow rates provide higher sensitivity than that with the higher flow rates of larger i.d. columns (for the columns having same separation efficiency, linear velocity, and same porosity).[9,27,28,33−35] Greater sensitivity using capillary electrophoresis (CE) with very small capillary diameters has been reported,[36] as well as "reduced elution speed" by using lower electrophoretic fields to decrease CE flow rates[37] and "peak parking" with LC separations to obtain a very low flow rate (<10 nL/min) to facilitate MS/MS analysis of peptides.[38] However, simply reducing the flow rate of separations reduces separation quality (due to both physical effects resulting from pressure changes and the decreased chromatographic performance at less than optimum mobile phase linear velocities[38]), and results in impractically long separation times if used through the entire separation.

LC separations that provide both high separation efficiencies and optimized ESI sensitivity are possible using sufficiently small i.d. columns, but doing so has presented a range of significant challenges involving: (1) sample injection to the columns, (2) the fabrication of the columns themselves, and (3) effective interfacing of the low flow rate column effluent with ESI-MS in a fashion that does not degrade the separation, provides high electrospray stability and generally robust operation. For example, we have previously reported on the use of 15-μm-i.d. packed particle LC columns providing flow rates of ~20 nL/min at an optimal LC linear velocity of 0.2 cm/s,[30] and enabling identification of peptides at low attomole levels using MS/MS with a conventional ion trap MS instrument.[30] However, effective preparation of small i.d. columns requires smaller diameter particles that are highly uniform and thus difficult to produce, making such columns difficult to pack, particularly for porous particles providing higher separation peak capacities.[39] Additionally, the low flow rates have required ESI emitter "tips" that are difficult to connect to the LC column in a fashion that does not degrade separation quality.[40] These issues have to date effectively precluded routine use or automated operation of <~50 to 75 microns i.d. LC columns. A capillary LC column with an integrated ESI emitter allows minimization of the postcolumn dead volume.[27,38,41,42]

In this study, we report the preparation of integrated 10-μm-i.d. silica-based porous monolithic LC columns and their initial application for more sensitive and quantitative proteome analyses using ESI-MS. We also describe the development of a microsolid phase extraction (microSPE) precolumn for ease of sample injection and cleanup prior to analysis and an integrated ESI emitter that enables robust automated LC in conjunction with nanoESI-MS analyses. Of particular note, the initial work demonstrates significantly improved detection limits (e.g., for MS/MS analyses), more effective quantitative measurements providing good linear correlations between MS responses and peptide amounts, as well as a more uniform response due to reduced ESI suppression and matrix effects. Finally, we provide an initial demonstration for the quantitation of immunopurified protein phosphatase 5 with validation using conventional methods.

Experimental Section

Sample Preparations. HeLa Tet-On cells (BD Biosciences Clontech, Palo Alto, CA) were used to generate a stable cell line expressing rat Flag-PP5 in a doxycyclin-inducible manner, according to the manufacturer's instructions. Cells were cultured in DMEM high glucose media with 10% FBS, 100 units penicillin/mL and 100 μg streptomycin/mL at 37 °C in a humidified atmosphere with 5% CO_2. Cells were either left untreated or treated with doxycyclin (2 μg/mL) for 48 h, washed twice with PBS and lysed in (100 mM NaCl, 50 mM Tris (pH 7.4), 15 mM EDTA, 15 mM EGTA, 10% glycerol, 0.1% Triton X-100, 10 mM NaF, 10 mM β-glycerophosphate, 2.5 mM Na_3VO_4, 10 nM microcystin-LR, 1 mM PMSF, 10 μg/mL benzamidine, and 1 μg/mL each of leupeptin, pepstatin, and chymostatin). Lysates were clarified by centrifugation and the supernatants incubated with EZview Red anti-FLAG M2 gel (Sigma, St. Louis, MO) for 1.5 h at 4 °C. The immune pellets were washed twice with lysis buffer lacking glycerol followed by two washes with (50 mM Tris, 150 mM NaCl, pH 7.4). Bound proteins were eluted with FLAG peptide (Sigma, St Louis, MO) in (50 mM ammonium bicarbonate, 150 mM NaCl) for 30 min at 4 °C.

Immunoprecipitated samples and recombinant Flag-PP5 were subjected to SDS-PAGE and silver stain or western blot analysis. PP5 was detected with monoclonal anti-PP5 (BD Transduction Laboratories, San Diego, CA) and goat anti-mouse secondary conjugated with Alexa Fluor 680 dye (Molecular Probes, Eugene, OR). Quantitation was performed using the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE). The concentration of PP5 in immunoprecipitated samples from control and induced cells was estimated using a standard curve generated from the fluorescence signal intensity of recombinant PP5 ranging from 10 to 80 ng. To digest the proteins in the IP samples, 1 μg modified trypsin was added, and the mixture was incubated overnight at 37 °C. Proteolysis was terminated by acidification of the reaction mixture with glacial acetic acid to ~pH 3.5.

In addition, both a bovine serum albumin (BSA, Sigma, St. Louis, MO) (0.1 mg/mL) and a *Shewanella* oneidensis[39] proteome tryptic digest (1.5 mg/mL) were used to evaluate the performance of the LC-ESI-MS.

Direct Infusion Studies of ESI−MS Response. A conventional ion trap mass spectrometer (Finnigan model LCQ XP, ThermoElectron Corp., San Jose, CA) was used with ESI

emitters pulled from 150-μm-o.d. × 50-μm-i.d. or 192-μm-o.d. × 100-μm-i.d. fused silica capillary using a butane microflame torch (Alltech Associates, Inc., Deerfield, IL) to provide different tip orifice i.d. for different flow rates. The temperature of the MS heated capillary inlet was fixed at 150 °C. During the measurements, both the electrospray voltage and the ESI emitter position were adjusted to provide maximum MS response at each flow rate, and a stereo microscope was used to monitor the electrospray stability. The MS responses of a mixture of three peptides [Human angiotensin I (AT), [Met-OH[11]]-substance P (SP), and vasoactive intestinal peptide (VA) (Sigma, St. Louis, MO)] at concentrations of 10^{-6} mol/L were measured at flow rates from 20 nL/min to 1 μL/min. MS scans with an m/z range of 150−2000 were acquired for 3 min starting 20 min after each flow rate change to ensure the flow rate had stabilized. The MS intensities reported for these three peptides represent the average intensities during this period of time.

Capillary LC-ESI-MS and LC-ESI-MS/MS. 10-μm-i.d. silica-based monolithic columns were prepared using protocols described previously.[29] A P-2000 laser puller (Sutter Instruments Co., Novato, CA) was used to pull uniform and reproducible emitter tips directly from the monolithic columns providing a tip i.d. of ∼1 μm and o.d. of ∼5 μm. The 25 cm ×10 μm i.d. monolithic columns provide flow rate of ∼10 nL/min at a pressure of 1000 psi (measured by connecting a 50 um i.d. open fused-silica capillary tubing to the outlet of the monolithic column and calculating the volume mobile phase that flowed through in a given time).

A fritted 360-μm-o.d. × 50-μm-i.d. fused-silica capillary (Polymicro Technologies, Phoenix, AZ) packed with 4 cm of 5-μm ODS-AQ particles (120-Å pores, YMC, Wilmington, NC) was used as the microSPE precolumn for sample injection/trapping. Because the analytical column is somewhat more hydrophobic than the SPE column (packed with more hydrophilic YMC ODS-AQ packing material[38]), peptides eluted from the SPE column are effectively "refocused" at the inlet of the analytical column, minimizing the effect of any dead volume between the SPE and the analytical column on separation quality. Samples were loaded onto the precolumn at ∼1000 psi at a flow rate of 0.5 μL/min. The coupling of a 50-μm-i.d. SPE column to the nanoscale LC increased the sample volume loading speed by ∼50-fold compared to a single-dimension LC column with the same i.d., enabling a 5-μL sample solution to be loaded in ∼10 min. In this initial off-line application, after washing with 0.1% acetic acid for 5 min, the precolumn was connected to the integrated 25 cm × 10 μm i.d monolithic column using 1 cm of 0.06-in. o.d. × 0.012-in. i.d. Teflon tubing (Zeus, Orangeburg, SC). The microSPE-LC assembly was attached to an HPLC system using a setup as reported previously.[38] Briefly, a PEEK tee served as a splitter. The gradient was delivered by an Agilent 1100 Series HPLC (Agilent Technologies, Santa Clara, CA). The microSPE-LC assembly was attached to the opposite arm of the splitter. PEEK tubing in the 90° arm of the splitter was first connected to a stainless steel union on which the ESI voltage (1.7 kV) was applied. Different lengths of 30-μm-i.d. fused silica capillaries were connected to the stainless steel union to adjust the mobile phase flow through the microSPE−LC assembly. By applying the ESI potential via a stainless steel union placed in the waste line, any bubbles and/or contaminants from the steel that may form at the electrical junction are flushed into the waste, resulting in a highly stable, low background.

Previously described procedures were used to pack the 150-μm-i.d. columns (5-μm ODS-AQ, 120-Å pores, YMC) for LC-MS studies.[33] A ESI emitter tip (30-um-i.d.) pulled from a 190-μm-o.d. × 75-μm-i.d. fused silica capillary was connected to the column outlet. The 25 cm × 150 μm i.d packed column was attached to a C2XH-0906 positive-feedback six-port switching valve (Valco Instruments, Houston, TX) with a 5-μL sample loop. ESI voltage (2.0 kV) was applied on the stainless steel union, which was used to connect the column and the ESI emitter.

A conventional ion trap MS (LCQ XP) or a linear ion trap MS (LTQ, ThermoElectron Corp) with a heated capillary temperature of 175 °C was used for sample analyses with on-line separations. For experiments using the LCQ, a scan time of ∼1.4 s (three microscans with a maximum ion injection time of 300 ms) over an m/z range of 400−2000 was used followed by MS/MS analysis of the three most abundant peaks from each MS scan. For experiments using the LTQ, a scan time of ∼0.15 s (one microscans with a maximum ion injection time of 10 ms) over an m/z range of 400−2000 was used followed by MS/MS analysis of the 10 most abundant peaks from each MS scan. A "collision energy" setting of 45% (for LCQ) or 35% (for LTQ) was applied for ion fragmentation, and dynamic exclusion was used to discriminate against previously analyzed ions (data-dependent analysis).

Quantitative Proteome Analyses. A very short reversed phase LC gradient (mobile phase B% from 0 to 100% in 17 min, with an effective separation time of approximately 7 min) was used with the bovine serum albumin (BSA) tryptic digest sample (to simulate the complexity with a long gradient for more complex samples in which many peptides coelute during separation). A BSA trypsin digest was first analyzed by LC-MS/MS to correctly assign peaks to BSA tryptic peptides, followed by LC-MS scans of m/z range 400−2000 for all the subsequent analyses. Peptide assignments were obtained using the SEQUEST program (ThermoElectron Corp.) according to previously established peptide identification criteria.[43] The chromatographic peak areas for BSA peptides from analyses with both the integrated 10-μm-i.d. monolithic column and the 150-μm-i.d. packed column were measured using the Xcaliber software, and the total peak area of peptides was derived from a summation of all peak areas for all ion charge states.

Results and Discussion

Analyses Using microSPE-10-μm-i.d. LC-ESI-MS Instrumentation. Silica-based Monolithic 25 cm long × 10-μm−i.d. capillary LC columns, providing an optimum flow rates of ∼10 nL/min, were fabricated with integral nanoESI emitters (i.e., from a single fused-silica capillary) and combined with a 50-μm-i.d. SPE precolumn to enable robust proteomics analyses of complex peptide mixtures. The sensitivity achievable for protein identification was initially evaluated using a bovine serum albumin (BSA) tryptic digest and conventional MS/MS analysis. As an example, a 5 attomole sample (BSA before digestion) in 1-μL solution was analyzed using the integrated monolithic column interfaced to a conventional ion trap (LCQ) for MS/MS. Multiple peptides were identified using the SEQUEST program and previously reported criteria. As an example of high sensitivity achieved with a much more complex mixture of peptides, analysis of 100 ng of a tryptic digest of soluble *Shewanella oneidensis* proteins[44] allowed 1332 proteins to be confidently identified (from 5164 different peptides) in a

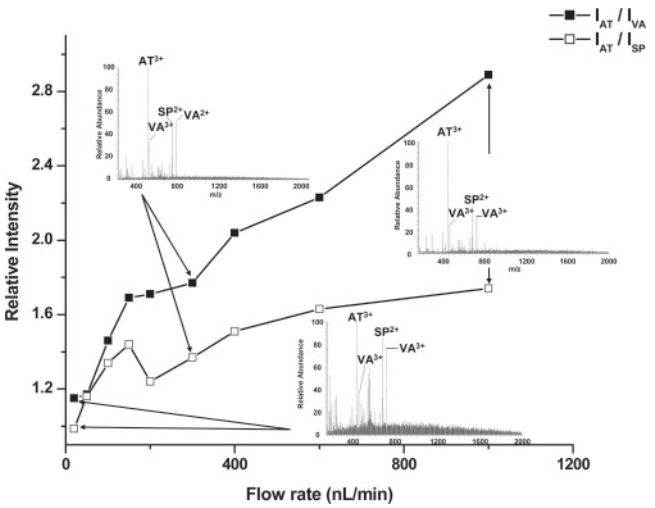

Figure 1. Ion intensity of Human angiotensin I (AT: 433.1(3+), 649.0(2+)) relative to that of vasoactive intestinal peptide (VA: 450.4(3+), 713.5(2+)) or [Met-OH[11]]-substance P (SP: 476.0(3+), 675.0(2+)) at different flow rates. Representative spectra at three different flow rates are also shown. Concentration: 10^{-6} mol/L for each peptide.

3 h analysis using a different linear ion trap (LTQ) for MS/MS (see Supporting Information).

More Quantitative Proteome Analyses at Lower Flow Rates. The ESI-MS response with flow rate for different peptides was illustrated by direct infusion of a mixture containing equimolar (10^{-6} M) concentrations of Human angiotensin I (AT), [Met-OH[11]]-substance P (SP), and vasoactive intestinal peptide (VA). The m/z and charge state of the dominant peptide ions were 433.1^{3+} (and to a lesser extent, 649.0^{2+}) for AT, 713.5^{2+} and 450.4^{3+} for VA, and 675.0^{2+} (and to a lesser extent, 476.0^{3+}) for SP, respectively. Figure 1 shows peak intensities of AT relative to VA and SP at different flow rates. At 1 μL/min AT has a greater intensity than VA or SP (a difference attributed to its greater surface activity), but ESI-MS responses for all three peptides become increasingly uniform as flow rate decreases, and nearly identical at ~20 nL/min. At this flow rate sensitivity is ~25-fold greater compared to 1 μL/min, but a more substantial background due to a wide range of low level species (often referred to as "chemical noise") is evident, attributed to more efficient ionization of species discriminated against using conventional ESI. While such chemical noise can limit the utility of direct ESI-MS when it interferes with detection of the species of interest (one persons noise is another's signal!), it is largely eliminated by the use of an LC separation with high purity mobile phases.

The ESI-MS response with a 10-μm-i.d. monolithic LC column was evaluated using a BSA tryptic digest and compared to a conventional 150 μm i.d. 5-μm ODS-AQ particles-packed column (flow rate of 1.5 μL/min, which is about the optimum flow rate for this column) providing similar LC peak widths. Figure 2A shows the peak areas for six of the more intense tryptic peptide peaks as a function of sample size for the 10-μm-i.d. monolithic LC column. The peak areas of the six peptides increased linearly up to 10−15 ng of digested BSA, and then begin to level off for larger samples. With the 150 μm i.d column, linear ESI-MS response was observed for sample loadings of up to ~150 ng of digested BSA for the same six peptides, with peptide peak areas leveling off and decreasing in some cases at higher levels (Figure 2B). Problematic sup-

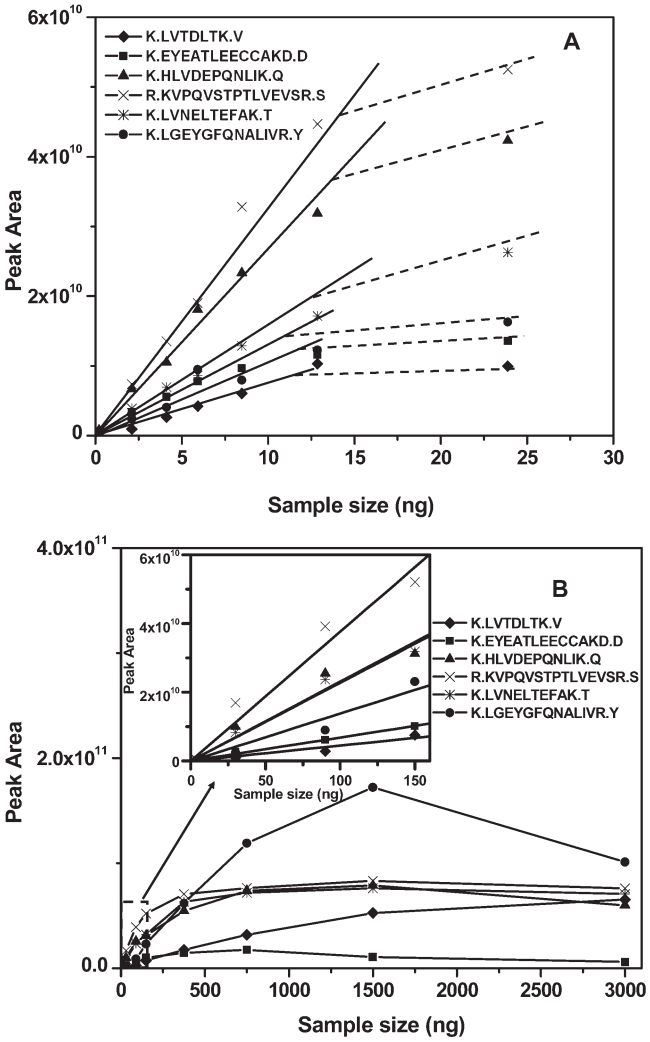

Figure 2. Peak area versus total amount of sample injected on to the column for six high abundance BSA tryptic peptides for a 25 cm × 10 μm i.d. monolithic column (A) and 25 cm × 150 μm i.d. packed column (B). Gradient: mobile phase B% from 0 to 100 in 17 min.

pression effects (as shown in Figure 2B) apply for the much larger range of sample loadings typically applied with larger i.d. columns; and thus, measurements for peptides having higher signal intensities, or coeluting with peptides having higher intensities, are less quantitative. The slopes of the linear response ranges for the peptides in Figure 2A vs B are 9- to 22-fold greater (Table 1), corresponding to the greater sensitivities obtained using the 10-μm-i.d. monolithic column, with the different gains attributed to the greater compound specific biases expected with the larger i.d. column. One may ask if there would be further gains with the use of even lower flow rate separations? Upon the basis of work at higher flow rates, a sensitivity gain of up to ~150-fold, rather than the 9- to 22-fold actually achieved, might have been anticipated due to the decrease in flow rate between 150 and 10-μm-i.d. columns. The likely reason for this achievable sensitivity gain is analyte limited, and ionization efficiencies are already close to 100%. If this explanation is correct, no further gains would be expected at lower flow rates using smaller i.d. LC columns. The presently reported monolithic columns have flow rates of ~10 nL/min (at an easily achieved pressure of 1000 psi for 25 cm

Table 1. Slopes of the Linear Fit (Figure 2) of Peak Area vs Sample Size for Six Peptides from an Albumin Tryptic Digest from LC-MS Analyses Using 10- and 150-μm-i.d. Columns[a]

	slope of linear fit ($\Delta_{\text{Peak Area}}/\Delta_{\text{Injection Amount}}$, $\times 10^7$)	
BSA peptide	10-μm i.d. column	150-μm i.d. column
K.LVTDLTK.V	110	5
K.EYEATLEECCAKD.D	160	7
K.HLVDEPQNLIK.Q	390	23
R.KVPQVSTPTLVEVSR.S	530	38
K.LVNELTEFAK.T	210	23
K.LGEYGFQNALIVR.Y	160	14

[a] Experimental conditions same as in Figure 2.

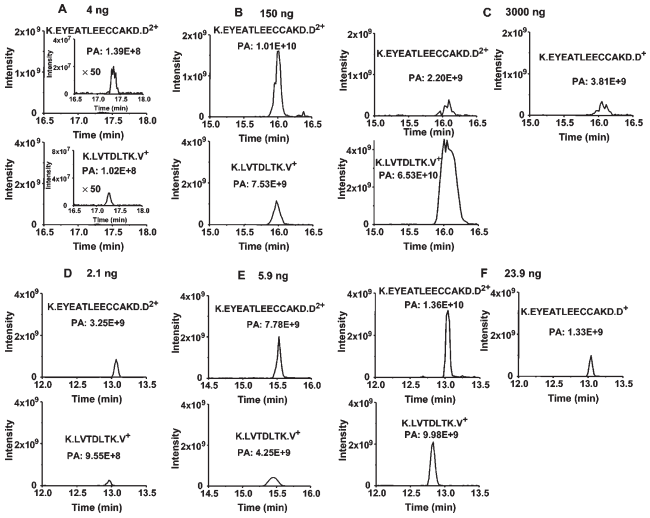

Figure 3. Extracted ion chromatograms for two BSA tryptic digest peptides (K.EYEATLEECCAKD.D^{2+} and K.LVTDLTK.V^{+}) coeluted from a 25 cm $\times$ 150 μm i.d. packed column when 4 ng (A), 150 ng (B), and 3000 ng (C) BSA tryptic digests were loaded, and from a 25 cm $\times$ 10 μm i.d. monolithic column with BSA tryptic digest loadings of 2.1 ng (D), 5.9 ng (E), and 23.9 ng (F), respectively. Note that the highest sample loading applied to both columns resulted in the detection of singly charged K.EYEATLEECCAKD.D^{+} peptide in addition to the strongly biased detection of K.LVTDLTK.V^{+}. Other conditions are the same as for Figure 2. PA: integrated LC-MS peak area.

long columns), a regime where the compound-to-compound variations in MS response are minimized and MS response is expected to vary more linearly with concentration.[9] The good linear relationships between peptide MS responses and sample amount for peptide tryptic digests are consistent with the expectation. While it is generally unavoidable that peptides will be produced with different efficiencies by tryptic digestion, minimization of variations in ESI response observed here (e.g., Table 1) improves, e.g., comparisons of the relative abundances of different proteins, and label-free approaches to quantitation in general.

Significantly different peptide specific responses observed at the higher LC flow rate limit the quantitative use of peak intensities for the range of sample loading typically used with conventional LC columns. Analyses with different sample loadings show numerous examples of peptides having nonlinear MS responses with the larger i.d. column. As an example, Figure 3 shows peaks for two coeluting peptides at 4 ng (A), 150 ng (B), and 3000 ng (C) BSA tryptic digest sample with the

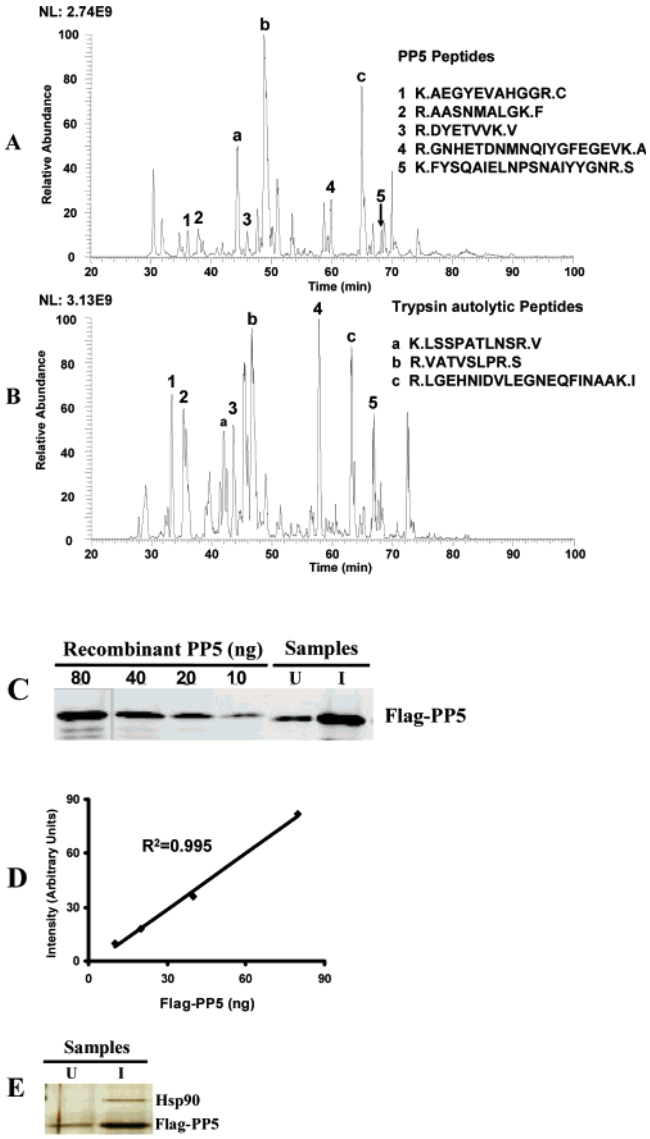

Figure 4. Base peak chromatograms from microSPE-LC-ESI-MS analyses of immunoprecipitated phosphatase 5 samples from either control (A) or induced (B) cells. Conditions: 1 μL control and induced IP samples were loaded on the precolumn for analyses, respectively. Column: 25 cm $\times$ 10 μm i.d. integrated silica-based monolithic column. Gradient: mobile phase A (H_2O, 0.1 mol/L acetic acid) to 50% B (80% ACN in mobile phase A) in 80 min with data collection initiated at the start of the gradient. Flow rate: $\sim$10 nL/min at a pressure of $\sim$1000 psi. Peaks 1–5 are for five PP5 peptides; a, b, and c are three trypsin autolytic peptides. SDS-PAGE and Western blot analysis of recombinant Flag-PP5 and immunoprecipitated Flag-PP5 in the uninduced *(U)* and induced *(I)* samples (C) and standard curve of recombinant Flag-PP5 within the range of 10–80 ng (D) along with silver stain gel showing the Hsp90 and Flag-PP5 bands in the uninduced *(U)* and induced *(I)* immunoprecipitate samples (E).

larger i.d. column. The data shows the response for K.EYEATLEECCAKD.D^{2+} to be suppressed by K.LVTDLTK.V^{+} at the higher sample concentration (Figure 3C), while more similar relative intensities are observed at lower level sample loadings (Figure 3A,B). The less quantitative nature of LC-ESI-MS analyses at conventional LC flow rates is a general problem that can plague measurements of both high and low abundance components, since it depends on the *total* analyte concentra-

Table 2. MicroSPE-LC-ESI-MS Analyses of Control and Induced Immunoprecipitated Flag-Tagged Protein Phosphatase 5[a]

		control						induced						
		peak areas ($\times 10^{10}$)						peak areas ($\times 10^{10}$)						
protein	peptide	run 1[b]	run 2[b]	run 3[b]	run 4[b]	ave[b]	RSD (%)	run 1[b]	run 2[b]	run 3[b]	run 4[b]	ave[b]	RSD (%)	$ave_{induced}/ave_{control}$
	K.AEGYEVAHGGR.C	0.57	0.74	0.58	0.63	0.63	13.7	3.01	2.90	4.41	2.33	3.16	27.9	5.0
	R.AASNMALGK.F	0.50	0.50	0.58	0.64	0.55	13	2.93	3.37	4.53	3.78	3.65	18.6	6.6
PP5	R.DYETVVK.V	0.93	1.00	1.06	1.09	1.02	6.98	4.76	6.07	6.70	6.42	5.99	14.3	5.9
	R.GNHETDNMNQIYGFEGEVK.A	1.20	1.26	1.64	1.46	1.39	14.4	7.12	8.11	8.05	6.91	7.55	8.23	5.4
	K.FYSQAIELNPSNAIYYGNR.S	0.38	0.63	0.92	0.99	0.73	38.2	2.48	2.57	3.7	5.63	3.6	40.8	4.9
	K.EDQTEYLEER.R	–[c]	–	–	–	–	–	0.28	0.37	0.40	0.42	0.37	17.5	–
HSP90	K.EQVANSAFVER.V	–	–	–	–	–	–	0.09	0.12	0.14	0.14	0.12	17.9	–
	K.ADLINNLGTIAK.S	–	–	–	–	–	–	0.10	0.13	0.17	0.26	0.17	42.7	–
	K.LSSPATLNSR.V	2.88	2.99	3.71	3.49	3.27	12.1	2.61	3.4	3.94	3.54	3.37	16.5	1.0
Trypsin[d]	R.VATVSLPR.S	7.55	8.71	10.1	10.2	9.14	13.8	8.66	10.1	10.6	10.3	9.93	8.75	1.1
	R.LGEHNIDVLEGNEQFINAAK.I	1.63	3.32	6.09	4.58	3.9	48.4	2.30	2.10	5.03	6.67	4.03	55	1.0

[a] Experimental conditions are the same as for Figure 4. [b] Peak areas measured as described in Methods. [c] Indicates that the peptides were not detected. [d] See text.

tion at any point in the separation. For this reason, the extent of ion suppression can often vary between analyses, depending upon the reproducibility of flow rate, the separation, and sample loading, and possibly also the ESI emitter performance.[13] Figure 3D,F shows the corresponding peaks for 2.1, 5.9, and 23.9 ng samples with the 10-μm-i.d. column. These data illustrate a more sensitive detection and reduced bias.

As an initial application we assessed levels of Flag-tagged protein phosphatase 5 (PP5) immunoprecipitated from HeLa cells either untreated or induced by doxycyclin. Using the 10-μm-i.d. monolithic LC column numerous peptides were identified by MS/MS from immunoprecipitated PP5 and Hsp90[45] (a PP5 protein partner), as well as trypsin. A total of 15 peptides from PP5 and none from Hsp90 were identified from the control sample, and 23 peptides from PP5 and 19 peptides from Hsp90 were identified from the induced sample. Figure 4A,B shows LC-MS base peak chromatograms of control and induced samples, and identifications for several peaks resulting from the MS/MS analyses. We also used trypsin autolytic peptides for an additional comparison of relative responses between analyses, based upon the assumption that they will have the same concentrations in both samples. Each sample was analyzed four times, and the peak areas for five PP5 peptides, three Hsp90 peptides, and three trypsin autolytic peptides from each analysis are given in Table 2. The results show the peak areas of the trypsin autolytic peptides in the control and induced samples are very similar for the two conditions. The peak area ratios of the five PP5 peptides from induced and control samples ranged from 4.9 to 6.6. Quantitative Western blot analysis of a portion of the same samples (Figure 4C,D), indicated that the immunoprecipitate from induced cells contained ~6-fold more Flag-PP5 than the untreated cells. In addition to the good agreement between the low flow rate LC-MS and Western blot data for Flag-PP5 quantitation, Hsp90 was observed in the immunoprecipitate from induced cells and not detected in the control sample by both LC-MS and by SDS-PAGE and silver stain analysis (Figure 4E).

Conclusions

The present microSPE-LC-ESI-MS arrangement provides an improved basis for quantitative proteomic analyses as an integrated part of a platform that provides much more robust sample injection and coupling with ESI-MS. The microSPE stage allows efficient sample introduction to the small diameter columns due to the larger diameter of the "precolumn" and the concomitantly higher flow rate that can be used for sample loading. The use of an integrated 10-μm-i.d. silica-based monolithic column has demonstrated good separation efficiencies, as well as greatly improved ESI-MS sensitivity compared to columns of conventional dimensions. Application of the high-sensitivity platform to the more complex *Shewanella oneidensis* tryptic digest provided identification of 1332 confident protein identifications from a single nanoLC-MS/MS analysis of 100 ng sample. The low nL/min flow rates from the 10 μm i.d. monolithic columns minimize the compound-to-compound variations in MS response and provide the basis for more quantitative measurements from "label free" analyses. As an example, we found the difference in protein level of immunopurified protein phosphatase 5 in control and induced cell samples measured by LC-MS to agree well with a quantitative fluorescence-based Western blot analysis. We anticipate a broad range of proteomics applications will benefit from the combination of improved sensitivity and "label free" methods for quantitation[8] that avoid most contributions due to ion suppression effect.

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Supporting Information Available: Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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