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On-Line 1D and 2D PLOT/LC-ESI-MS Using 10 μm i.d. Poly (styrene–divinylbenzene) Porous Layer Open Tubular (PLOT) Columns For Ultrasensitive Proteomic Analysis

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Abstract

Following on our recent work, on-line one dimensional (1D) and two dimensional (2D) PLOT/LC-ESI-MS platforms using 3.2 m $\times$ 10 μm i.d. poly(styrene-divinylbenzene) (PS-DVB) porous layer open tubular (PLOT) columns have been developed to provide robust, high performance and ultrasensitive proteomic analysis. Using a PicoClear tee, the dead volume connection between a 50 μm i.d. PS-DVB monolithic microSPE column and the PLOT column was minimized. The microSPE/PLOT column assembly provided a separation performance similar to that obtained with direct injection onto the PLOT column at a mobile phase flow rate of 20 nL/min. The trace analysis potential of the platform was evaluated using an in-gel tryptic digest sample of a gel fraction (15 to 40 kDa) of a cervical cancer (*SiHa*) cell line. As an example of the sensitivity of the system, ~2.5 ng of protein in 2 μL solution, an amount corresponding to 20 *SiHa* cells, was subjected to on-line microSPE-PLOT/LC-ESIMS/MS analysis using a linear ion trap MS. 237 peptides associated with 163 unique proteins were identified from a single analysis when using stringent criteria associated with a false positive rate less than 1%. The number of identified peptides and proteins increased to 638 and 343, respectively, as the injection amount was raised to ~45 ng of protein, an amount corresponding to 350 *SiHa* cells. In comparison, only 338 peptides and 231 unique proteins were identified (false positive rate again less than 1%) from 750 ng of protein from the identical gel fraction, an amount corresponding to 6000 *SiHa* cells, using a typical 15 cm $\times$ 75 μm i.d. packed capillary column. The greater sensitivity, higher recovery, and higher resolving power of the PLOT column resulted in the increased number of identifications from only ~5% of the injected sample amount. The resolving power of the microSPE/PLOT assembly was further extended by 2D chromatography *via* combination of the high-efficiency reversed phase PLOT column with strong cation exchange chromatography (SCX). As an example, 1071 peptides associated with 536 unique proteins were identified from 75 ng of protein from the same gel fraction, an amount corresponding to 600 cells, using 5 ion exchange fractions in online 2D SCX-PLOT/LC-MS. The 2D system, implemented in an automated format, led to simple and robust operation for proteomic analysis. These promising results demonstrate the potential of the PLOT column for ultratrace analysis.

Global characterization of proteins from complex mixtures over a wide dynamic concentration range is one of the challenges of current proteomic studies.¹ Multidimensional high performance liquid chromatography (HPLC) has received considerable attention for such analyses.²⁻³ Among the several LC combinations, coupling strong cation exchange (SCX) as

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the first dimension and reversed phase (RP) as the second dimension of separation is often used.⁴⁻⁸ Current existing 2D approaches are typically operated with relatively large amounts of sample (low microgram); however, the study of much smaller sample amounts (e.g., limited number of cells obtained from laser capture microdissection) are difficult.⁹⁻¹⁰ New 2D LC/MS approaches with improved sensitivity and robust operation are thus highly desirable.

Low nanoLC separation (<50 nL/min flow rate) coupled with electrospray ionization-mass spectrometry (ESI-MS) provides significant advantages over higher flow LC, including increased sensitivity and reduced ion suppression.¹¹⁻¹⁷ Several issues must be addressed for nanoLC-ESI-MS to be a robust tool for sensitive analysis, including: 1) production of reproducible high efficiency ultra-narrow bore LC columns, 2) effective, low dead volume on-line coupling of a microSPE column to the nanoLC for ease of sample loading and cleanup, while not degrading separation. Although recent successful efforts have been made, there are difficulties associated with the preparation of small i.d. LC columns (<25 μm). For example, 10 μm i.d. LC columns packed with 1.0 μm particles generated extremely high pressure ($\sim 30,000$ psi/10 cm long column at a linear velocity of 0.4 cm/s)¹⁸. In addition, while significant efforts have recently been made in the preparation of narrow bore monolithic (silica and organic polymer) capillary columns^{15,17, 19-22}, the production of reproducible and robust 10 and 20 μm i.d. monolith columns still remains a challenge. Minimization of dead volume in the system is also difficult, given that peak volumes are generally in the low to mid nanoliter range.

Porous layer open tubular (PLOT) LC columns with capillaries smaller than 20 μm have in the past been examined with limited success.²³⁻³⁰ Polymer-based PLOT columns are attractive because of their phase ratio relative to open tubular columns, their high stability and ease of preparation.³⁰⁻³² 20 μm i.d. poly(butyl methacrylate-coethylene dimethacrylate)-based PLOT column have been prepared for high efficiency CEC separation.³³

Our laboratory recently developed an *in-situ* polymerization method to prepare long, high efficiency poly(styrene-divinylbenzene) (PS-DVB) 10 μm i.d. PLOT columns in a single step, following the coating of the walls of the capillary.³⁴ The PSDVB PLOT columns demonstrated good reproducibility (3% RSD column-to-column retention time) and relatively high loading capacities for 10 μm diameter columns (~ 100 fmol for angiotensin). Attomole to sub-attomole detection limits were achieved at a flow rate of 20 nL/min when the PLOT column was coupled to ESI-MS. In our previous work, we loaded the sample onto a short 50 μm i.d. PS-DVB monolithic microSPE column. After loading, the microSPE column was inverted and butt-to-butt connected to the PLOT column using a PicoClear union. A peak capacity of ~ 400 was achieved from the off-line microSPE-PLOT/LC-ESI-MS analysis.

In this work, we describe new on-line 1D and 2D PLOT/LC-ESI-MS/MS platforms that use a 3.2 m $\times$ 10 μm i.d. PLOT column for ultratrace proteomic analysis. The dead volume at the microSPE column/separation column connection was minimized by using a newly designed PicoClear tee to realize high efficiency LC separations at the low flow rate of 20 nL/min. The platform is demonstrated to provide a practical, robust system for operation of 10 μm PLOT columns with high sensitivity and resolving power for proteomic analyses at the ultratrace level.

EXPERIMENTAL

Materials

Fused silica capillary tubing was purchased from Polymicro Technologies (Phoenix, AZ). Styrene, divinylbenzene, ethanol, formic acid (HPLC grade), 3-(trimethoxysilyl)propyl methacrylate, 2, 2'-diphenyl-1-picrylhydrazyl (DPPH), *N,N*-dimethylformamide anhydrous

(DMF), tetrahydrofuran (THF), 2, 2'-azobisisobutyronitrile (AIBN), ammonium bicarbonate, dithiothreitol (DTT), and iodoacetamide (IAA) were obtained from Sigma-Aldrich (St. Louis, MO). Ammonium acetate, acetonitrile (HPLC grade), and deionized water (HPLC grade) was from Fisher Scientific (Fair Lawn, NJ). A standard tryptic digest of bovine serum albumin (BSA) was purchased from Michrom Bioresources Inc. (Auburn, CA). Trypsin (sequencing grade) was from Promega (Madison, WI). The *SiHa* cervical cancer cell line was obtained from American Type Culture Collection (ATCC) (Manassas, VA, USA).

1D RPLC-ESI-MS

10 μm i.d. PS-DVB PLOT columns were prepared using procedures described previously.³⁴ Briefly, a degassed solution containing 5 mg of AIBN, 200 μL styrene, 200 μL divinylbenzene, and 600 μL ethanol was filled into a 10 μm i.d. capillary pretreated with 3-(trimethoxysilyl) propyl methacrylate. Both ends of the capillary were sealed with septa, and the capillary was heated at 74 $^{\circ}\text{C}$ for ~ 16 h in a water bath. The column was then washed with acetonitrile and was ready for use. The two ends of the capillary were sealed in water when the column was not in use. In addition, 50 μm i.d. PS-DVB monolithic microSPE columns were prepared using a procedure modified from ref(20). A low density PS-DVB monolithic column, 4 cm $\times$ 50- μm -i.d., was synthesized from a polymerization solution containing 5 mg of AIBN, 200 μL styrene, 200 μL divinylbenzene, 40 μL THF, and 550 μL of decanol. The column provided a flow rate of ~ 1 $\mu\text{L}/\text{min}$ at a back pressure of ~ 2900 psi, for rapid sample loading.

To evaluate the efficiency of the PLOT column, the column was carefully butt-to-butt connected to a coated spray tip (360 μm o.d., 20 μm i.d. fused silica with a 5 μm i.d. spray tip, 2–3 cm length, New Objective, Woburn, MA) with a PicoClearTM connector (New Objective), with the electrospray voltage being applied directly on the spray tip. A PEEK tee (Upchurch Scientific Inc., Oak Harbor, WA) was used as a splitter. The sample was loaded onto the PLOT column directly at a split ratio of 1:1000.

MicroSPE column enrichment is a necessary procedure for handling samples of a few microliters volume. A PicoClear tee (New Objective) was used to minimize the dead volume between the microSPE and PLOT column (see Figures 1). Samples were loaded onto the microSPE column either by split loading (Figure 1A) or direct loading (Figure 1B). A C18 reversed phase capillary column (15 cm $\times$ 75 μm i.d., Magic C18, 3 μm particle size, 200 $\text{\AA}$ pore size, Michrom Bioresources, Auburn, CA) was used to compare with the PLOT column. The flow rate was ~ 200 nL/min for direct sample loading and separation on the latter column. Gradient elution was performed using an Ultimate 3000 Pump System (Dionex, Sunnyvale, CA) with mobile phase A as 0.1% (v/v) formic acid in water and mobile phase B as 0.1% (v/v) formic acid, 10% (v/v) water in acetonitrile.

2D SCX-PLOT/LC-ESI-MS

A 100 μm i.d. fused-silica capillary packed with 5 μm 300 $\text{\AA}$ Polysulfoethyl ATM strong cation-exchange resin (Nest Group Inc., Southboro, MA) was used for the first dimension separation. The SCX column was directly connected to a second 6-port injection valve (see Figure 1C) using a screen embedded liner with nanovolume ferrules (Valco Instruments, Houston, TX). The buffers used to form the salt gradient steps (5 – 250 mM) were prepared from a 1 M ammonium acetate stock solution in solvent A, containing 5% acetonitrile.

Mass spectrometry—NanoESI-MS was performed on an LTQ ion trap mass spectrometer (Thermo Fisher, San Jose, CA). The data generated from the LC/MS experiments were analyzed using the CPAS system³⁵ with a Sequest Cluster search engine (ver. 3.0). The database search was conducted against a human protein database consisting of SwissProt entries (release 52 with 15945 protein sequences) with combined normal and reversed

sequences to facilitate the estimation of the false positive rate.³⁶ Trypsin was specified as the digestion enzyme with one missed cleavage and carboxyamidomethylation of cysteines was designated as a fixed modification of cysteines. Peptides were assigned based on a Peptide Prophet probability³⁷ ≥ 0.95 , $\Delta Cn \geq 0.10$, and $Xcorr \geq 1.8$, 2.5 and 3.75 for singly, doubly, and triply charged ions, respectively. By employing these stringent criteria, peptides were identified with an estimated false positive rate of less than 1%.

Sample preparation—A tryptic digest sample of BSA and an in-gel tryptic digest of a lysate of *SiHa* cells were used as test mixtures to evaluate the performance of the PLOT/LC-ESI-MS platform. In-gel digestion of *SiHa* cell protein extraction was performed using protocols described previously.³⁸ A total of 6×10^6 *SiHa* cells were lysed with 2% SDS in 50 mM NH_4HCO_3 via five bursts of 20-second sonication followed by 20-second ice cooling. 0.4% of the protein extract ($\sim 8 \mu\text{g}$ of total protein, an amount corresponding to 24,000 cells) was loaded on an SDS PAGE gel (4% – 12% gradient) for separation. After electrophoresis, the gel was cut into 3 sections with molecular weight ranges less than 15 kDa (section 3), between 15 kDa and 40 kDa (section 2), and greater than 40 kDa (section 1). Following reduction with dithiothreitol (DTT) and alkylation with iodoacetamide (IAA), an 8 ng/ μL trypsin solution (pH 8.0) was added and incubated overnight at 37°C. The supernatant was removed and saved. Gel pieces were further extracted with 5% formic acid (200 μL) in an extraction buffer (acetonitrile: 50 mM NH_4HCO_3 = 2:1) at 37°C for 15 minutes. The formic acid solution, containing tryptic peptides, was combined with the previous supernatant and concentrated to 10–20 μL ($\sim 3 \mu\text{g}$ of protein per gel section) using a SpeedVac. The sample prepared from the 15 to 40 kDa gel fraction (0.30 $\mu\text{g}/\mu\text{L}$ of protein, a concentration corresponding to 2400 cells/ μL) was then diluted between 8 – 120 fold for use in the study.

RESULTS AND DISCUSSION

The dead volume between a microSPE column and the separation column must be minimized in order to maintain the separation performance of a nanoLC column. Several approaches, including back-flush elution²⁴, “band refocusing” (using an analytical column which is more hydrophobic than the microSPE column)^{30,39}, and manually connecting the microSPE column to the analytical column using Teflon tubing⁴⁰ or a PicoClear union³⁴, have been developed to minimize loss of separation performance. However, on-line coupling of a microSPE column with an ultra-narrow bore 10 μm i.d. LC column operated at very low flow rate ($< 30 \text{ nL}/\text{min}$) can still be a challenge.

In our previous paper, we employed an off-line microSPE-PLOT/LC-ESI-MS system for ultrasensitive proteomic analysis. The sample was first loaded off-line onto a 50 μm i.d. PS-DVB monolithic microSPE column. The microSPE column was inverted and manually coupled to the PLOT column using a PicoClear union. The sample was then injected onto the PLOT column in the back-flush mode and gradient elution conducted. The system demonstrated high resolving power at a flow rate of 20 nL/min . In this study, a 4 cm $\times$ 50 μm i.d. PS-DVB low density monolithic microSPE column was constructed and coupled on-line to the PLOT column using a newly designed PicoClear tee. The most important feature of the tee is that it can minimize the dead volume at the SPE column/PLOT column connection and hold pressure up to 5000 psi. Figure 1 presents the various designs incorporated in this work, with the monolithic microSPE column being butt-to-butt connected to the PLOT column through visual inspection (see insert in Figure 1A). Note that the capillary ends must be carefully cut vertically to make the dead volume negligible and to ensure that the mobile phase can be easily directed to waste during the sample loading step. A 75 μm capillary, connected to the PicoClear tee in the 90° arm, was carefully positioned close to the microSPE/PLOT joint.

On-line 1D PLOT/LC-ESI-MS analyses

We first evaluated the ability of the high pressure stable PicoClear tee as a means of connecting the microSPE column to the PLOT column for on-line sample loading and clean up. In the first experiment, 2 nL (800 amol) BSA tryptic digest was directly loaded onto a $3.2 \text{ m} \times 10 \text{ } \mu\text{m}$ i.d. PLOT column at a split ratio of 1:1000. The gradient separation of the BSA tryptic digest on the PLOT column was then performed, yielding the results shown in Figure 2A. A high efficiency separation was observed, similar to that found in our previous work.³⁴ Next, the tee was used to connect the PLOT column to a $4 \text{ cm} \times 50 \text{ } \mu\text{m}$ i.d. PS-DVB monolithic microSPE column, see Figure 1A. The fused silica capillary connected to the PicoClear tee in the 90° arm was plugged, such that flow was not allowed to pass through this capillary. Using the same split ratio of 1:1000, 2 nL of sample was trapped onto the microSPE column upon loading, followed by forward-elution onto the PLOT column. Gradient elution separation of the identical 800 amol BSA tryptic digest sample, after loading on the microSPE column, is shown in Figure 2B. Little or no loss in separation relative to direct PLOT column loading is observed, as the average peak widths at half height are 4.6 and 4.9 seconds in Figure 2A and 2B, respectively. Thus, it is apparent that the dead volume at the microSPE / PLOT column connection was minimized sufficiently such that little or no measureable degradation of the separation on the PLOT column at the very low flow rate of 20 nL/min was observed. It should be noted that, as a result of the difference in the gradient delay in the two chromatograms in Figure 2, the peptide retention time was shifted in Figure 2B, relative to that in Figure 2A. In addition, there was an increase in the effective separation window since the microSPE/PLOT column system was more retentive than the PLOT column alone. Nevertheless, since the monolithic microSPE column used similar chemistry as for the PLOT column (PS-DVB), there was minimal change in elution order, and the peak shapes were very sharp in the two chromatograms. It is interesting to note that several commercially available, traditional “zero dead volume” tees were also examined to connect the PLOT column to the microSPE. Significantly broader peaks were observed when the similar sample and experimental procedures were employed (data not shown). These results demonstrate that the PicoClear tee is a good means of column connection at the extremely low flow rate.

From the promising results in Figure 2, we then designed an on-line microSPE-PLOT/LC-ESI-MS system for efficient loading and analysis of a practical volume of sample, see Figure 1B. Here, the entire 2 μL volume in the sample loop could be loaded onto the microSPE column at a flow rate of $\sim 1 \text{ } \mu\text{L}/\text{min}$ and the trapped sample then injected on-line to the $3.2 \text{ m} \times 10 \text{ } \mu\text{m}$ i.d. PLOT column for separation. The 10-port valve was first set in the loading position, with tee **a** serving as splitter and tee **b** as connector. The sample solution was pumped through the microSPE column, while salts and other ESI deleterious components were directed to waste through a $75 \text{ } \mu\text{m}$ i.d. fused silica capillary connected to the PicoClear tee (solid line in Figure 1B). After loading, the 10-port valve was switched to the separation position, and the microSPE column was positioned to be on-line with the PLOT separation column. The tees were thus reversed with the first tee now serving as connector, while the second acted as splitter. In addition, the mobile phase flow was split immediately before the microSPE/PLOT assembly to minimize the gradient delay. Samples trapped on the microSPE column were forward-eluted onto the PLOT column for reversed phase LC separation.

The configuration in Figure 1B provided several potential advantages. Given the extremely low flow rate in the PLOT column ($\sim 20 \text{ nL}/\text{min}$), mobile phase splitting immediately before the microSPE/PLOT assembly was critical to minimize the gradient delay volume. In addition, the microSPE column served as a particulate filter to minimize clogging of the PLOT column or the ESI emitter. All salts and other impurities in the sample solution were directed to waste prior to reaching the PLOT column. Additionally, because the injection valve was washed at a relatively high flow rate, sample carryover was greatly reduced. Most importantly, separation

with the microSPE column was achieved with a performance equivalent to that acquired with the separation column alone.

The in-gel tryptic digest sample of a 1D SDS gel fraction of the cervical cancer cell line (*SiHa*), was used to test the on-line microSPE-PLOT/LC-MS platform. The sample prepared from the 15 to 40 kDa fraction (0.30 $\mu\text{g}/\mu\text{L}$ of protein, a concentration corresponding to 2400 cells/ μL) was then diluted between 8 – 120 fold for use in the study. While this sample is artificial since it has been made by dilution from a large number of cells, it can nevertheless provide a measure of the detection sensitivity and resolving power of the PLOT/LC-MS platform. A total of 237 unique peptides and 163 proteins were found from ~ 2.5 ng of protein in 2 μL solution, an amount corresponding to 20 *SiHa* cells, using a linear ion trap MS. The average numbers of peptides and proteins identified from triplicate analysis of the identical sample were 241 (RSD 10.6%) and 160 (RSD 8.1%), respectively. Among them, 130 peptides (54%) and 105 proteins (66%) were identified from all of the three analyses. The fact that more peptides were not observed in all three runs is most probably due to data dependent scanning. Figure 3 shows that the number of identified peptides and proteins increased significantly up to 638 and 343, respectively, as the injected protein amount was raised from 2.5 to 45 ng, the amount corresponding to 20 and 350 cells, respectively. In this study, we used a combined normal and reversed protein database for assessing the false positive rates of peptide identifications. By employing the stringent criteria described in the Experimental Section, peptides were confidently identified with a false positive rate less than 1%.³⁶

The performance of the on-line microSPE-PLOT/LC-ESI-MS system was further compared with that of a typical 15 cm $\times$ 75 μm i.d. packed capillary column. An in-gel tryptic digest of 750 ng of protein from the gel fraction (15 to 40 kDa), an amount corresponding to 6,000 *SiHa* cells, was directly loaded onto the 75 μm packed column, followed by nanoLC-ESI-MS analysis at a flow rate of 200 nL/min with an identical gradient as that for the PLOT column. Based again on a false positive rate of less than 1%, the packed column resulted in the identification of 338 unique peptides and 231 distinct proteins in a single analysis, using the linear ion trap MS (LTQ). Overall, roughly twice as many peptides were identified from 45 ng of protein using the on-line microSPE-PLOT/LC-ESI-MS system relative to that from 750 ng of protein using the packed capillary column. Figure 4 shows in detail the comparison between the two analyses. The greater sensitivity, higher recovery, and higher resolving power of the PLOT column³⁴ are the likely reasons for the greater number of identifications from only 1/20 the sample amount injected. The results demonstrate the high sensitivity and performance capabilities of the platform in Figure 1B. Obviously, when handling such small sample amounts, there are challenges with respect to minimizing losses; however, once the sample is loaded on the microSPE column, low detection limits are readily achievable.

On-line 2D SCX-PLOT/LC-ESI-MS analysis using high-efficiency 10 μm PLOT column

The resolving power of LC-MS can be greatly extended by the combination of high-efficiency RPLC with a second orthogonal dimension of separation, e.g. strong cation exchange (SCX). However, using ultranarrow bore LC columns at very low flow rates in on-line 2D LC separation is challenging. As shown in Figure 1C, an on-line 2D SCX-PLOT/LC-ESI-MS platform was constructed by adding an additional six port valve to the platform of Figure 1B. Samples for 2D LC separation were first loaded onto the SCX column, and the peptides that were unretained on the SCX column were trapped onto the 50 μm i.d. PS-DVB monolithic microSPE column. As above, the non-retained sample components on the monolithic microSPE column were directed to waste through a 75 μm fused silica capillary connected to the PicoClear tee in the 90° arm. After completion of SCX loading, the second 6-port valve and the 10-port valve were then switched to the separation position (dashed line in Figure 1C), in order to couple the monolithic microSPE column to the PLOT column for reversed phase

LC separation. When the gradient separation of the unretained SCX fraction was complete, both the second 6-port and 10-port valves were switched back to the loading position. The series of 5 steps of increasing ammonium acetate concentration the first 6-port injection valve were then sequentially pumped into the SCX column. After each step, the eluted peptides from the SCX column were trapped onto the monolithic microSPE column, with the salt and buffer being diverted to waste. The monolithic microSPE column was then washed with excess solvent A, and the gradient LC separation, as described above, was performed.

Figures 5A–F show the base peak chromatograms of the SCX flow through sample plus the 5 ammonium acetate steps for the analysis of 75 ng of total protein from the identical gel fraction (15 to 40 kDa), an amount corresponding to 600 *SiHa* cells, and Figure 6 presents the distribution of peptides and proteins identified from each fraction. The number of identified peptides and proteins were found to be highest in fractions 1 and 2, with lower amounts in the remaining fractions. In total, 1071 peptides covering 536 unique proteins were identified, representing 68% and 56% more, respectively, than peptides and proteins found from the single 1D PLOT/LC-ESI-MS analysis (see Figure 3). In a duplicate 2D SCX-PLOT/LC-ESI-MS analysis of the identical sample, 1222 peptides associated with 675 proteins were identified. Among them, 634 peptides (55%) and 375 proteins (62%) were identified from both analyses, again due to data dependent scanning. Given that the sample was only an in-gel digest of a single gel band from such a small amount of total protein, these results are highly promising in demonstrating the potential of the current platform for high-resolution analysis at the ultratrace level,

CONCLUSION

An automated 1D and 2D PLOT/LC-ESI-MS platform using 10 μm i.d. columns has been demonstrated for high resolution ultratrace proteomic analysis. The platform is shown to be sensitive, highly resolving and robust. The PLOT column was operated at the flow rate of 20 nL/min, which made it impractical in terms of time to load sample volumes larger than a few hundred nanoliters. In order to overcome this limitation, we implemented a microSPE/PLOT column assembly to handle μL volumes of sample in a reasonable time while maintaining the separation performance essentially equivalent to that obtained with direct loading on to the PLOT column. The forward-elution arrangement provided an additional advantage over our previous “back-flush” design in that the 50 μm i.d. PS-DVB monolithic trap column served as a “filter” to minimize clogging of the PLOT column and ESI emitter. Application of the high efficiency and high sensitivity platform to in-gel tryptic digests sample of a gel fraction of *SiHa* cells provided identification of more than 1.5 times as many as proteins from a single nanoLCMS/MS analysis in comparison to twenty-fold greater amount of sample using a typical 75 μm i.d. packed capillary column. In a subsequent 2D SCX-PLOT/LC-MS analysis of the same sample, more than one thousand peptides covering five hundred different proteins were identified from 75 ng of protein, the amount corresponding to roughly 600 cells. Work is continuing on the tissue-specific proteomic analyses of a limited number of cells obtained from laser capture microdissected (LCM) samples.

Finally, it can be noted that while in this study we have focused mainly on the 2D SCX-PLOT/LC-MS system with very low mobile phase flow rate, the optimized platform is clearly also applicable for coupling the PLOT column with other functional LC columns in automated formats (e.g. immobilized metal ion affinity chromatography (IMAC) and lectin affinity). Thus, a variety of multidimensional LC separations can be readily implemented with the 10 μm PLOT column.

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Figure 1.

Schematic diagrams of the high efficiency 1D (Figure 1A and 1B) and 2D (Figure C) PLOT/LC-ESI-MS systems using a $3.2 \text{ m} \times 10 \text{ } \mu\text{m}$ i.d. PLOT column. Fused-silica capillary packed with strong cation-exchange resin was used for sample fractioning. (A) Sample was loaded onto the microSPE column ($50 \text{ } \mu\text{m}$ i.d. PS-DVB monolith) by split loading, (B) The entire $2 \text{ } \mu\text{L}$ sample was loaded onto the microSPE column directly at a flow rate of $\sim 1 \text{ } \mu\text{L}/\text{min}$, and the trapped sample was then switched on-line to the $3.2 \text{ m} \times 10 \text{ } \mu\text{m}$ i.d. PLOT column for separation.; (C) Sample was first loaded onto an SCX column (Polysulfoethyl ATM), and then stepwise eluted and trapped onto the RP microSPE column. The trapped sample was switched

on-line to the PLOT column for separation. Full details can be found in the Experimental Section.

Figure 2.

Gradient PLOT/LC-ESI-MS of a mixture of a tryptic digest of BSA using a $3.2 \text{ m} \times 10 \text{ } \mu\text{m}$ i.d. PS-DVB PLOT column. Gradient: mobile phase A (0.1% (v/v) formic acid in water) to 40% B (0.1% (v/v) formic acid, 10% (v/v) water in acetonitrile) in 45 min with data collection initiated at the start of the gradient. Flow rate: $\sim 20 \text{ nL/min}$ at an inlet pressure of $\sim 2300 \text{ psi}$. (A) 800 attomole of the mixture was directly loaded on the PLOT column by split injection; (B) 800 attomole of the mixture was first loaded onto a $4 \text{ cm} \times 50 \text{ } \mu\text{m}$ i.d. PS-DVB monolithic microSPE column by split injection, the loaded sample was then forward-eluted onto the PLOT column.

Figure 3. Number of unique peptides and proteins identified from various sample amounts of in-gel tryptic digest of a gel fraction (15 to 40 kDa) of *SiHa* cells, using a microSPE and PLOT column. The amount of samples injected corresponded to 20, 75, 175, and 350 *SiHa* cells, respectively. Other conditions are the same as in Figure 2.

Figure 4.

Venn diagram of the number of peptides (A) and proteins (B) identified by the microSPE-PLOT/LC-ESI-MS analysis of ~45 ng of protein from a single gel fraction, an amount corresponding to 350 *SiHa* cells, with the nanoLC-ESI-MS analysis of the same gel fraction of ~750 ng of protein, an amount corresponding to 6,000 *SiHa* cells, using a conventional 15 cm × 75 μm i.d. packed column (PC). Experimental conditions for microSPE-PLOT/LC-ESI-MS analysis are the same as in Figure 2. NanoLC-ESI-MS was performed at a flow rate of 200 nL/min using a C18 reversed phase capillary column (Magic C18, 3 μm particle size, 200 Å pore size).

Figure 5.

High efficiency on-line 2D SCX-PLOT/LC-ESI-MS/MS analyses of an in-gel tryptic digest sample of a SDS-PAGE section of ~75 ng of protein, an amount corresponding to 600 *SiHa* cells: (A) Initial breakthrough LC-MS/MS analysis; (B - F) LC-MS/MS analyses after each salt step elution at ammonium acetate concentration of 5, 10, 15, 20, and 250 mM, respectively. 2 μ L of ammonium acetate solution was delivered to the SCX column for each salt step elution. Other conditions are the same as in Figure 2.

Figure 6. Distribution of unique peptides and proteins identified from each fraction of the 2D SCX-PLOT/LC-ESI-MS analysis of ~75 ng of protein, an amount corresponding to 600 *SiHa* cells. See Figure 5 for conditions.