analytical chemistry

Technical Note

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Integrated Platform for Proteome Profiling with Combination of Microreversed Phase Based Protein and Peptide Separation via Online Solvent Exchange and Protein Digestion

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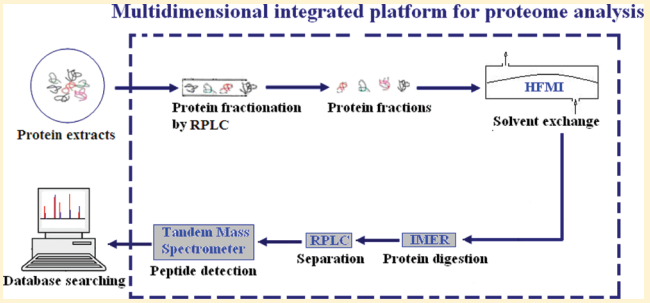
Supporting Information

ABSTRACT: An online integrated platform for proteome profiling was established, with the combination of protein separation by microreversed phase liquid chromatography (μRPLC), online acetonitrile (ACN) removal, and pH adjustment by a hollow fiber membrane interface (HFMI), online digestion by an immobilized enzymatic microreactor (IMER), as well as peptide separation and proteins identification by μRPLC or nano-RPLC-electrospray ionization tandem mass spectrometry (μRPLC-ESI-MS/MS). To evaluate the performance of such a platform, a three-protein mixture with mass ranging from 5 to 500 ng was analyzed automatically. Compared to the offline counterpart, although similar protein sequence coverages were obtained by the integrated platform, the signal intensity of total ion chromatogram was improved by almost 4 times. In addition, such an integrated platform was further applied for the analysis of extracted proteins from rat brain. Compared to the results obtained by offline counterpart and traditional MudPIT approach under similar conditions, by the integrated platform, the identified protein group number was comparable, but the analysis time was shortened to less than half of that taken by the traditional approaches. All these results demonstrated that our developed integrated platform might offer a promising tool for high-throughput and large-scale profiling of proteomes.



With the acceleration of proteome research, the development of novel approaches for high efficient and high throughput analysis of proteins becomes urgent. Recently, considerable efforts have been devoted to the development of nongel-based proteome technologies through the combination of various chromatography and electrokinetic separation methods with mass spectrometry (MS) or tandem MS analysis.[1−5] Among them, two-dimensional high performance liquid chromatography-MS/MS (2D-HPLC-MS/MS) is one of the most prospective approaches, as it provides high resolving power, versatility, sensitivity, and automation. However, despite the great progress that was made, not even the most efficient 2D-HPLC-MS/MS system is currently able to adequately separate the huge number of peptides resulting from the digestion of whole proteomes. To decrease the complexity of proteome samples, protein prefractionation is often performed before peptide separation. Currently, most reported protein prefractionation approaches are performed offline. However, offline operation might suffer from sample loss, time-consuming operation, and difficulty in automation.[6−8] Therefore, an ideal platform for proteome analysis should be composed of online protein separation, digestion, peptide separation, and identification.

Fast digestion is a crucial step to online integrate protein and peptide separation. Several approaches have been attempted to increase the efficiency of conventional in-solution proteolysis by high pressure,[9,10] microwave irradiation,[11] infrared radiation[12,13] and laser radiation,[14] but the online hyphenation of in-solution digestion protocol with HPLC or capillary electrophoresis (CE) is rather difficult. To solve these problems, much attention has been paid to the development of immobilized enzymatic reactors (IMERs).[15−19] In recent years, numerous immobilized trypsin reactors were developed, and coupled with HPLC-MS/MS or CE-MS/MS to achieve high throughput analysis of proteomes.[20−22] However, without protein fractionation before IMERs, the complexity of proteomes limits the identification capacity of such partially integrated platforms.

Since trypsin works well at weak basic conditions (pH 8.0−8.5), to match such conditions, in our previous work, WAX/

Received: August 19, 2011
Accepted: April 21, 2012
Published: April 23, 2012

   dx.doi.org/10.1021/ac3006796 | *Anal. Chem.* 2012, 84, 5124−5132

WCX or SEC separation for online protein prefractionation were coupled with IMER-HPLC-MS/MS and improved proteome profiling was obtained.[23,24] However, for protein separation, RPLC is more efficient.[25] The challenges of coupling RPLC with IMER come from the incompatibility of eluents with high concentration organic modifier and low pH value. Recently, to solve these problems, Schriemer et al. developed an integrated platform with combination of protein separation by RPLC, online dynamic dilution by a tee joint, online protein digestion by IMER, and direct peptide detection by MS.[26] Although to some extent the incompatibility of eluents from RPLC with IMER could be overcome by such a tee joint, the lack of trapping proteins or peptides before or after the IMER might lead to the decreased sample concentration, and thus the decreased MS detection sensitivity. Therefore, the hollow fiber membrane device, developed and utilized for protein desalting[27] and buffer exchange,[28] might be a promising interface to establish RPLC-based integrated platforms

In this paper, an integrated platform with the combination of protein separation by μRPLC, online acetonitrile (ACN) removal, and pH adjustment of eluents by a hollow fiber membrane interface (HFMI), online digestion by an IMER, and peptide separation and protein identification by μRPLC or nano-RPLC-ESI-MS/MS was established. Under the optimal conditions, the integrated platform was applied for the analysis of protein extracts from rat brain. Compared to traditional approaches, the platform demonstrated advantages of high efficiency, high throughput, and ease of automation, indicating that such integrated platforms might provide an attractive alternative for proteome profiling.

EXPERIMENTAL SECTION

Chemicals and Materials. Cytochrome c (horse heart, cyt-c), trypsin (bovine pancreas), β-lactoglobulin (bovine milk, β-lac), and protease inhibitor cocktail were purchased from Sigma (St. Louis, MO). Bovine serum albumin (BSA) was obtained from Sino-American Biotechnology (Luoyang, China). HPLC-grade ACN was bought from Merck (Darmstadt,Germany). Tetrathoxysilane (TEOS, 95%), 3-amino-propyltriethoxysilane (ATPES, 99%), and sodium cyanoborohydride were from Acros Organics (Geel, Belguim). Water was purified by a Milli-Q system (Millipore, Bedford, MA). All other chemicals and solvents were analytical-grade.

Sample Preparation. Standard proteins (cyt-c, BSA, and β-lac) were first denatured in 50 mM NH_4HCO_3 buffer (pH 8.0) containing 8 M urea for 1 h at 37 °C in a water bath, and then reduced by 5 μL of 1 M dithiothreitol (DTT) at 56 °C for 1.5 h. After cooling to the room temperature, 10 μL of 1 M iodoacetamide (IAA) was added to the solution, and the reaction was performed at room temperature in the dark for 40 min.

Rat brain tissue was cut into small pieces, then washed with ice-cold PBS three times, and finally ground to powder in liquid nitrogen. The powder was suspended in the extraction buffer composed of 8 M urea and 1% (v/v) protease inhibitor cocktail. The suspension was first homogenized for 60 s at 10 000 rpm, then ultrasonicated for 100 s at 130 w, and finally centrifuged at 25 000 rpm for 40 min. The supernatant was collected and precipitated overnight with ten volumes of ice-cold acetone. After centrifugation at 20 000 rpm for 30 min at 4 °C, the pellet was collected, and lyophilized by a Speed Vac Concentrator (Thermo, San Jose, CA), then redissolved in 8 M urea. The protein concentration was determined by BCA assay. The obtained protein extracts were reduced by DTT at 56 °C for 1.5 h, and alkylated by IAA at room temperature in the dark. Finally, the protein solution was stored at −20 °C before use.

Protein Digestion. After dilution with 50 mM NH_4HCO_3 buffer (pH 8.0) to decrease urea concentration below 1 M, BSA (0.1 mg/mL, 1 mL) and protein extracts from rat brain (0.1 mg/mL, 1 mL) were in solution digested by adding trypsin into pretreated samples with a substrate-to-enzyme ratio of 50:1 (m/m) and 25:1 (m/m), followed by incubation at 37 °C, respectively, for 12 and 20 h. Finally, 2 μL formic acid was added into the solution to terminate the reaction.

The resulting peptides were desalted with the following procedures: a C18 trap column (4.6 mm i.d × 10 mm, 5 μ, 120 Å) connected to an HPLC pump (Shimadzu Corporation, Japan) was first activated with 80% ACN containing 0.1% TFA and then equilibrated with 2% ACN containing 0.1% TFA at the flow rate of 1 mL/min. Then samples were loaded on the trap column and washed with 2% ACN containing 0.1% TFA at 1 mL/min to elute salts. Finally, peptides were eluted with 1 mL of 80% ACN and then dried at low temperature using a Speed Vac Concentrator. The pellets were collected and stored in the refrigerator under −20 °C for further use. Without specific statement, all percentages represented volume percentage.

Preparation of Organic−Inorganic Hybrid Silica Monolith-Based Microreactor. The organic−inorganic hybrid silica monolith-based immobilized trypsin microreactor was prepared according to our previous protocol.[29] In brief, the capillary with inner diameter of 250 μm was filled with polymerization solution containing TEOS (112 μL), APTES (118 μL), andydrous ethanol (215 μL), cetyltrimethyl ammonium bromide (8 mg), and water (32 μL) and then placed into a 40 °C water bath for 24 h to form an organic−inorganic monolith. Subsequently, the monolithic support was activated by flushing a solution of 10% glutaraldehyde for 6 h at room temperature, and trypsin was covalently bonded by continuously pumping 2 mg/mL trypsin dissolved in 100 mM phosphate buffer (pH 8.0) containing 50 mM benzamidine and 5 mg/mL sodium cyanoborohydride for 24 h at 4 °C. After the microreactor was purged with 1 M Tris-HCl (pH 8.0) and 20% acetonitrile (ACN), respectively for 4 h, the microreactor was filled with 0.02% (w/v) NaN_3 solution and stored at 4 °C before use.

For offline evaluation of the IMER, 0.1 mg/mL BSA was pumped through the microreactor at the flow rate of 1 μL/min by a syringe pump and the resulting products were manually collected and then analyzed by MALDI-TOF/MS.

Preparation and Evaluation of HFMI. The membrane interface was prepared according to our previous approach,[27] but with some modifications. Briefly, a length of about 10 mm of polyimide coating of fused silica capillaries of 100 μm i.d. × 375 μm o.d. (inlet capillary) and 75 μm i.d × 375 μm o.d. (outlet capillary) were first removed by a blade, and then respectively etched by 50% HF at room temperature until the capillaries could be inserted into a 65 mm-long hollow cellulose acetate fiber membrane (200 μm i.d., cutoff molecular weight of 3000 Da), which was taken from a capillary dialyzer (GFS Plus 12, Gambro Dialysatoren GmbH, Hechingen, Germany). Then they were glued together via epoxy glue, threaded through a centrifugal tube (10 mL), and fixed with two pieces of Teflon tubes. Finally, two holes were drilled on the two opposite sides of the centrifugal tube to introduce exchange buffer. To avoid

dx.doi.org/10.1021/ac3006796 | Anal. Chem. 2012, 84, 5124−5132

nonspecific protein adsorption, the inner surface of inlet capillary and outlet capillary of membrane interface were respectively treated with polyacrylamide coating and amido grafting.[30]

The performance of the membrane interface was evaluated by the analysis of BSA digests. At first, the BSA digests were trapped on a C18 precolumn (XBP C18, 1.0 mm i.d × 35 mm, 5 μm, 200 Å), and then, the solution containing 80% ACN was pumped through the interface at the flow rate of 2 μL/min into the C18 precolumn, with 50 mM NH_4HCO_3 buffer (pH 8.3) applied simultaneously as exchange buffer at the flow rate of 2 mL/min. Finally, the residual peptides on the C18 precolumn were separated by a μRPLC column (XBP C18, 0.3 mm i.d × 50 mm, 5 μm, 200 Å) and detected by ESI-MS/MS. The mobile phases for peptide separation were H_2O containing 0.1% formic acid (A) and ACN containing 0.1% formic acid (B); the analyses were performed at the flow rate of 5 μL/min. The gradient was set as follows: 5% (0 min) → 2% (10 min) → 15% (15 min) → 40% (75 min) → 80% (80 min) B. After each μRPLC separation, the column was equilibrated with the initial mobile phase for 20 min.

Protein Analysis by Offline Method. For standard protein analysis, a three-protein mixture, composed of 5 ng cyt-c, 50 ng β-lac, and 500 ng BSA, was first separated by a μRPLC column (Hypersil C8, 0.3 mm i.d × 150 mm, 5 μ, 300 Å) at a flow rate of 5 μL/min. The solvents for μRPLC based protein separation were H_2O containing 0.1% TFA (A) and ACN containing 0.1% TFA (B). The gradient was set as follows: 5% (0 min) → 5% (30 min) → 80% (30.1 min) → 80% (80 min) B. Protein fractions (250 μL) were collected offline and dried at low temperature using a Speed Vac Concentrator. The pellets were redissolved in 50 mM NH_4HCO_3 buffer (pH 8.0) and in solution digested (37 °C, 12 h), with a substrate-to-enzyme ratio of 50:1 (m/m), and the resulting peptides were separated by μRPLC column (XBP C18, 0.3 mm i.d × 150 mm, 5 μm, 200 Å) at the flow rate of 5 μL/min and detected by ESI-MS/MS. The mobile phases for peptide separation were H_2O containing 0.1% formic acid (A) and ACN containing 0.1% formic acid (B). The gradient was set as follows: 5% (0 min) → 15% (5 min) → 40% (65 min) → 80% (70 min) → 80% (75 min). After each μRPLC separation, the column was equilibrated with the initial mobile phase for 20 min.

For analysis of protein extracts from rat brain, 10 μg protein extracts were injected into a μRPLC column (Jupiter C4, 0.3 mm i.d × 150 mm, 5 μ, 300 Å), and six organic solvent steps were applied to elute proteins at the flow rate of 10 μL/min, including 25% B, 30% B, 35% B, 40% B, 50% B, and 80% B. Each protein fraction was collected offline and dried at low temperature using a Speed Vac Concentrator. The pellets were redissolved in 50 mM NH_4HCO_3 buffer (pH 8.0) and digested in solution (37 °C, 20 h), with a substrate-to-enzyme ratio of 25:1 (m/m), and the resulting peptides were separated by a nano-RPLC column (XBP C18, 0.075 mm i.d × 150 mm, 5 μm, 200 Å) at the flow rate of 300 nL/min and detected by ESI-MS/MS. The gradient was set as follows: 2% (0 min) → 15% (5 min) → 40% (95 min) → 80% (100 min) → 80% (105 min). After each nano-RPLC separation, the column was equilibrated with the initial mobile phase for 20 min.

Protein Analysis by Traditional MudPIT. For MudPIT based analysis of extracted proteins from rat brain, proteins were first digested in solution, and desalted following the procedure described in the Protein Digestion section. A 10 μg portion of protein digests were redissolved in 2% ACN containing 0.1% formic acid and then analyzed by 2D-SCX-nanoRPLC-ESI/MS/MS. For the first-dimension separation, an SCX column (0.25 mm i.d × 30 mm, 10 μm, 1000 Å) was used, with 0.1% formic acid, and 1000 mM ammonium acetate, with pH adjusted to 3 with formic acid as buffers. Six salt steps, including 10, 50, 100, 150, 200, and 500 mM, were applied at the flow rate of 10 μL/min, and peptides were eluted stepwise by 300 μL of each elution solvent. The resulting peptides were then captured and separated by a nano-RPLC column (XBP C18, 0.075 mm i.d × 150 mm, 5 μm, 200 Å) at the flow rate of 300 nL/min under the same conditions applied for the offline method. Finally the eluents were detected by ESI-MS/MS. After each nano-RPLC separation, the column was equilibrated with the initial mobile phase for 20 min.

Proteins Analysis by Integrated Platform. For standard protein analysis, the same 3-protein mixture was first separated by a μRPLC column under the same conditions applied for the offline method, and then the eluted protein fractions were passed through an HFMI (0.2 mm i.d × 65 mm) to remove online ACN and adjust pH. To speed up such a procedure, 50 mM NH_4HCO_3 (pH 8.3) containing 5% ACN was used as the exchange buffer at the flow rate of 2 mL/min. Subsequently, proteins were digested online by IMER (0.25 mm i.d × 50 mm) at the flow rate of ca. 1 μL/min. The resulting peptides were then captured by a precolumn (XBP C18, 1.0 mm i.d × 35 mm, 5 μm, 200 Å) and separated by an XBP C18 column (0.3 mm i.d × 150 mm, 5 μm, 200 Å) under the same conditions applied for the offline method.

For the analysis of proteins extracted from rat brain, a 10 μg sample was injected, and the same six organic solvent steps were applied to elute proteins from a μRPLC column (Jupiter C4, 0.3 mm i.d × 150 mm, 5 μ, 300 Å) at a flow rate of 10 μL/min, each fraction from RPLC was passed through an HFMI (0.2 mm i.d × 65 mm) to remove ACN and adjust pH with 50 mM NH_4HCO_3 (pH 8.3) containing 5% (v/v) ACN at the flow rate of 2 mL/min as the exchange buffer. Subsequently, the protein fraction was digested online by IMER (0.25 mm i.d × 50 mm) at a flow rate of ca. 1 μL/min. The resulting peptides were captured by an XBP C18 precolumn (0.25 mm i.d × 10 mm, 10 μm, 100 Å) and separated by a nano-RPLC column (XBP C18, 0.075 mm i.d × 150 mm, 5 μm, 200 Å) at the flow rate of 300 nL/min under the same conditions applied for the offline method, followed by the identification by ESI-MS/MS. After each nano-RPLC separation, the column was equilibrated with the initial mobile phase for 20 min.

MS Identification. An ultraflex III MALDI-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) with a polished target was utilized for offline protein identification. A 7 mg/mL CHCA dissolved in a 2:1 mixture of ACN/H_2O, containing 0.1% (v.v) TFA, was used as a matrix. MALDI-TOF MS experiments were performed with constant laser intensity in positive ion mode. All mass spectra were obtained with an accumulation of 1200 laser shots in reflector mode with an accelerating voltage of 21.85 kV.

An LTQ-XL ion trap mass spectrometer (Thermo Electron, San Jose, CA) was hyphenated with a μRPLC/nano-RPLC for online protein identification. If not specially stated, the spray voltage of MS was set at 3.2 KV, and the temperature of ion transfer capillary was 250 °C. The MS/MS collision energy was set at 35%. During μRPLC/nano-RPLC-MS/MS analysis, the effluents were sprayed directly into ESI source using a commercial interface. All MS and MS/MS spectra were

dx.doi.org/10.1021/ac3006796 | Anal. Chem. 2012, 84, 5124−5132

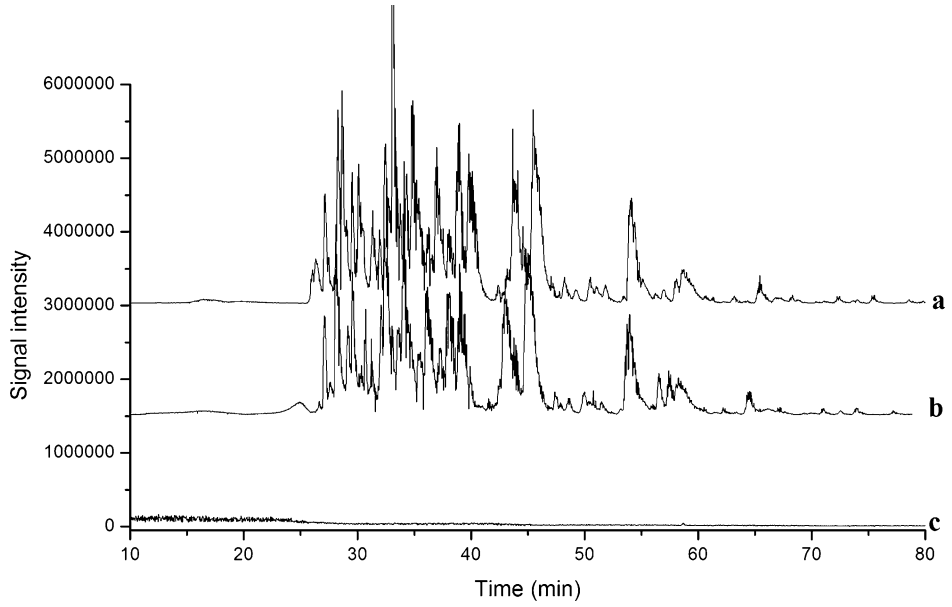


Figure 1. Base peak chromatograms of BSA digests analyzed by μRPLC-MS/MS: (a) direct sampling; (b) captured by C18 precolumn, then eluted by 80% ACN exchanged by HFMI; (c) captured by C18 precolumn, then eluted by 80% ACN. Experimental conditions were shown in the Experimental Section.

acquired in the data-dependent mode, by which MS acquisition with the mass range of m/z 400−1800 was automatically switched to MS/MS acquisition with the automated control of Xcalibur software. The ten most intense ions of the full MS scan were selected as the parent ions and subjected to MS/MS scan with an isolation width of m/z 2.0. The dynamic exclusion function was set as follows: repeat count 2, repeat duration 30 s, and exclusion duration 180 s.

Database Searching. For MALDI-TOF MS analysis, all acquired MS spectra were searched against SwissProt database (517802 sequences) using MASCOT (version 2.3.2). Trypsin was set as enzyme for database searching, maximum two missed cleavage sites were allowed, cabamidomethyl (C) was set as fixed modification, and oxidation (M) was set as variable modification; mass peptide tolerance was set as ±100 ppm.

For HPLC-MS analysis, all MS/MS spectra acquired in one raw file were converted to single *.mgf file using pExtract software,[31] and then, the *.mgf file was searched against SwissProt database and the International Protein Index (IPI) rat database (v3.75, 79 426 entries) using Mascot server (v2.3.2) (Matrix Science, Boston, US). Trypsin was set as the enzyme for database searching. Peptides were searched using fully tryptic cleavage constraints and up to two missed cleavage sites were allowed. Cysteine residues as a static modification of 57.0215 Da, and methionine residues as a variable modification of +15.9949 Da were searched. The mass tolerances were 2 Da for parent ions and 1 Da for fragment ions. Peptides from standard proteins with significant threshold P value less than 0.05 (rank 1) and expected cut off value (E-value) = 0.05 were used for protein identification. For the identification of proteins extracted from rat brain, reversed sequences were appended to the database to evaluate the false discovery rate (FDR), and the Mascot searching results were aligned by pBuild[31] to reduce the apparent redundancy and control FDR < 1% in protein identification. When the same peptides were assigned to multiple proteins, the assigned proteins were clustered into a "protein group". To further compare different approaches, only protein group with distinct peptides ≥2 was analyzed.

RESULTS AND DISCUSSION

For proteomic analysis, it is important to improve the analysis efficiency, throughput, and automation. Therefore, in this work, an online integrated platform combining protein separation by μRPLC, online solvent exchange by an HFMI, online digestion by an organic−inorganic hybrid monolith-based immobilized trypsin reactor, peptide separation, and identification by μRPLC or nano-RPLC-ESI-MS/MS is proposed.

Improvement on Compatibility of μRPLC Based Protein Separation and IMER. Compared to SEC and IEC, RPLC offers higher efficiency for protein separation.[32] In most cases, protein separation by RPLC is performed at low pH (with TFA added) with increased organic solvent concentration in the mobile phase. Therefore, to establish a RPLC-based integrated platform for proteome profiling, it is indispensable to keep good compatibility of protein fractions eluted from the RPLC column with the trypsin immobilized microreactor, with the optimal digestion efficiency at pH 8.3 and ACN content lower than 20%.[33] Herein, a hollow fiber membrane with cutoff molecular weight of 3000 Da was used to prepare the interface to remove ACN and adjust pH of each protein fraction, with 50 mM NH_4HCO_3 buffer (pH 8.3) as the exchange buffer.

The ACN removal capacity of HFMI was first evaluated by online treatment of 80% ACN solution with 0.1% TFA as exchange buffer at the flow rate of 2 mL/min. The eluents from the outlet of HFMI were collected, and residual ACN concentration was determined to be ca. 2% according to a previous reported protocol[28] (see Supporting Information Figure S-1).

Furthermore, through the analysis of BSA digests captured on a C18 precolumn, which were respectively eluted by 80% ACN without or with HFMI as exchange interface, it could be also seen that similar to that obtained by direct analysis of BSA digests under the same conditions, with sequence coverage of 69% (Figure 1a), with HFMI as an interface for ACN removal, BSA could be identified with the sequence coverage of 67% (Figure 1b). However, without ACN removal by HFMI, almost none peptide could be detected (Figure 1c). All these results

dx.doi.org/10.1021/ac3006796 | Anal. Chem. 2012, 84, 5124−5132

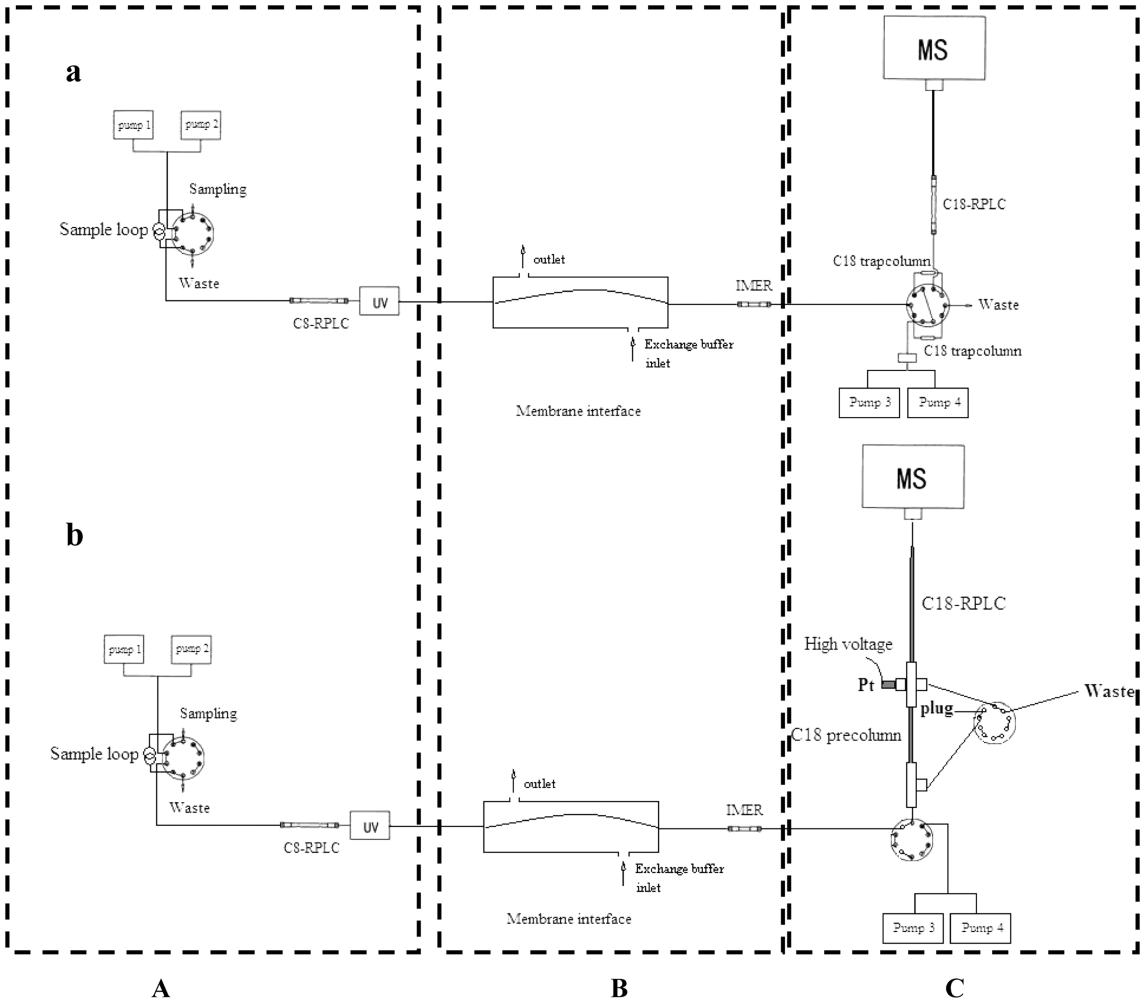


Figure 2. Schematic diagram of integrated platforms for proteome analysis: (a) μRPLC-HFMI-IMER-μRPLC-ESI-MS/MS; (b) μRPLC-HFMI-IMER-nano-RPLC-ESI-MS/MS; (A) protein separation; (B) online protein pretreatment; (C) peptide analysis.

demonstrated that the developed HFMI could effectively remove high concentration ACN.

In addition, the pH adjustment capacity of HFMI was evaluated by continuously introducing 0.1% TFA into the interface, which was simultaneously adjusted with 50 mM NH_4HCO_3 buffer (pH 8.3) as the exchange buffer. The obtained pH value of eluants from outlet of HFMI was measured as about 8.0, compatible with tryptic digestion, demonstrating the good pH adjustment capacity of such an interface.

Fast Protein Digestion by IMER. To achieve online integrated analysis, fast proteolytic digestion is another crucial step. In our previous works, various IMERs with proteases covalently bound to different supports, such as polymer monolith,[34] polymer particle,[23] and hybrid silica monolith,[29] were developed and showed good promise to achieve online integration. With consideration on the fact that the back-pressure generated from IMER might destroy the pore structure of HFMI, resulting in protein loss, it was indispensable to select an IMER with high enzymatic activity and low back pressure. Herein, according to our previous study, an organic−inorganic hybrid silica monolith based immobilized trypsin microreactor was utilized, which was proven of high surface area for trypsin immobilization and macroporous structure with good permeability.[35]

The enzymatic activity of such an IMER (0.25 mm I.D × 50 mm) was evaluated by the digestion of 0.1 mg/mL BSA at room temperature, which was pumped at the optimal flow rate of 1 μL/min. With 200 ng of digests analyzed by MALDI-TOF MS, the sequence coverage of 67.3 ± 1.2% (n = 3) was obtained, which demonstrated that the online protein digestion could be achieved by such an IMER with high efficiency and good reproducibility (see Supporting Information Table S-2).

The operational stability of such an IMER was also investigated. In our experiments, 0.1 mg/mL BSA was continuously digested with IMER at the flow rate of 1 μL/min, and 30 μL of the digests was collected between each run. With 200 ng of digests analyzed by MALDI-TOF MS, the average sequence coverage was 57.6% with the RSD as 4.3% (n = 20). Furthermore, the lifetime of IMER was studied by the digestion of 0.1 mg/mL BSA at the flow rate of 1 μL/min in 7 consecutive days, BSA could be still digested sufficiently by the IMER in the seventh day with the comparable sequence coverage (61 ± 2.8%, n = 3). These results demonstrated that such an IMER could endure long time usage and be competent for the establishment of an integrated platform.

***μ*RPLC-HFMI-IMER-*μ*RPLC-ESI-MS/MS Platform.** The schematic diagram of the μRPLC-HFMI-IMER-μRPLC-ESI-MS/MS platform was shown in Figure 2a. To synchronize the online treatment of protein eluates from μRPLC column by

dx.doi.org/10.1021/ac3006796 | Anal. Chem. 2012, 84, 5124−5132

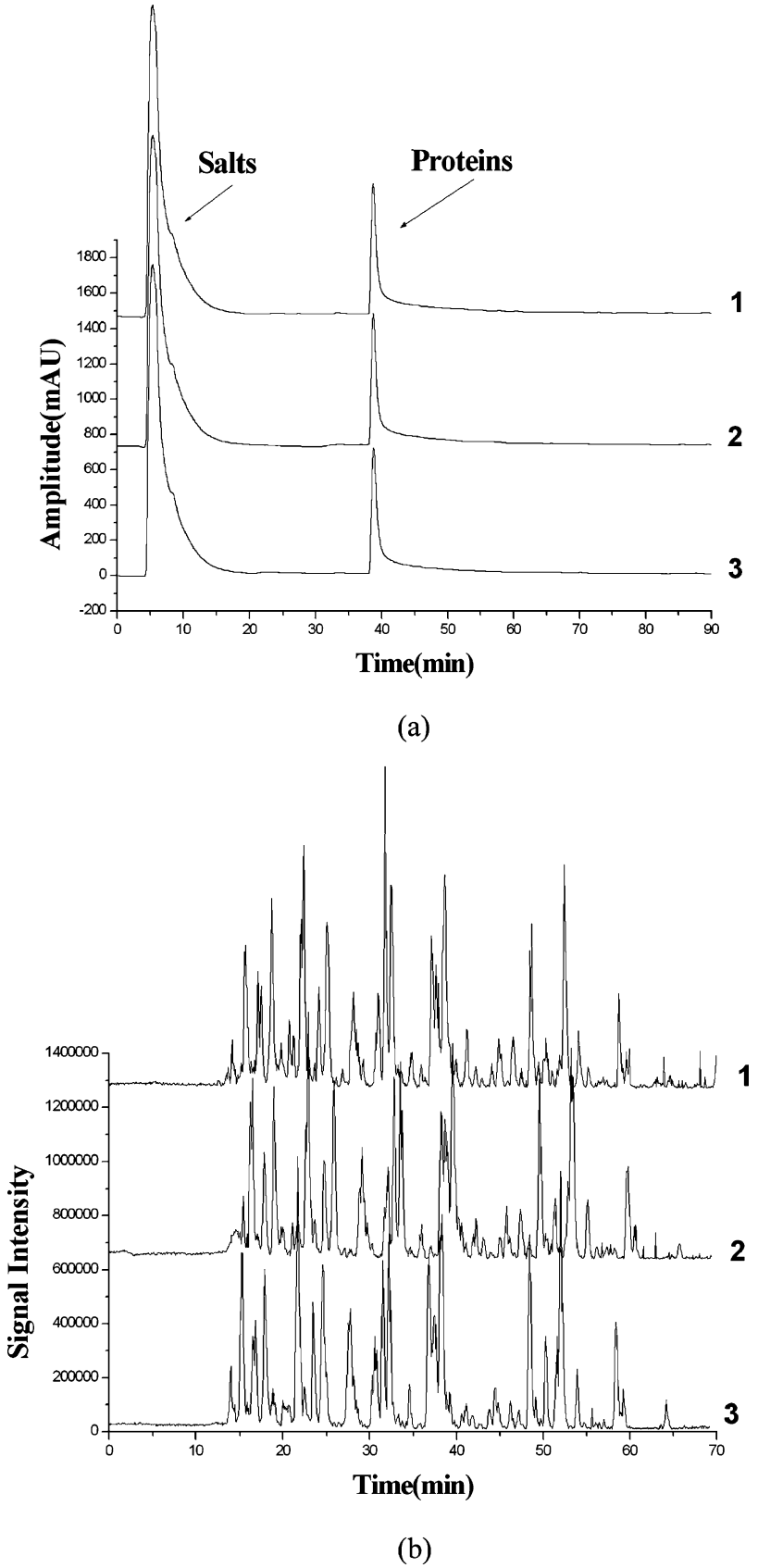


Figure 3. Reporducibility of (a) three-protein mixture eluted from μRPLC by 80% ACN; (b) Base peak chromatograms of three-protein mixture analyzed by μRPLC-HFMI-IMER-μRPLC-ESI/MS/MS integrated platform in triplicated runs. Experimental conditions were shown in the Experimental Section.

dx.doi.org/10.1021/ac3006796 | *Anal. Chem.* 2012, 84, 5124−5132

HFMI and IMER with peptide separation, C18 precolumns (1.0 mm I.D. × 35 mm) were used for capturing protein digests, and the protein sample was divided into several fractions with a constant time interval. In addition, due to the back pressure of IMER and C18 precolumns, the flow rate of protein eluates from μRPLC was decreased from 5 to about 1 μL/min, compatible with optimal flow rate for protein digestion by IMER.

Such an integrated platform had three advantages: (1) Proteins were first fractionated before being digested into peptides, which significantly decreased the challenges for peptide separation, consequently enhanced the accuracy of protein identification by reducing the complexity of samples. (2) Protein fractions from μRPLC were treated online by HFMI and IMER, helpful to shorten sample preparation time, and reduce the possibility of sample loss or contamination. (3) The platform could be operated automatically, ensuring the good reproducibility for proteome profiling.

To investigate the performance of such an integrated platform, a three-protein mixture, composed of 5 ng cyt-c, 50 ng β-lac, and 500 ng BSA, with p*I*s ranging from 4 to 9, was analyzed. The chromatograms of protein separation and base peak chromatograms of peptide separation in triplicated runs were shown in Figure 3. By database searching, the sequence coverages for these proteins were respectively 10%, 65%, and 78%. To compare with integrated platform, the same sample was also analyzed by offline protein prefractionation by μRPLC, in-solution digestion, and peptide analysis by μRPLC-ESI-MS/MS under the same separation conditions. By database searching, with the same strict threshold, the obtained sequence coverages for these proteins were 10%, 55%, and 81%, respectively, comparable to those obtained by integrated platform (see Supporting Information Table S-1). However, by the integrated platform, not only the total analysis time was shortened from 16 to 4 h, but also the signal intensity of total ion chromatogram was improved by almost 4 times (as shown in Supporting Information Figure S-2), which demonstrated that by the integrated platform, both sample loss and time consumption, could be reduced. Furthermore, the RSDs of sequence coverage for cyt-c, β-lac, and BSA obtained by the integrated platform were 0%, 2.3%, and 6.8% (n = 3), demonstrating the good operation reproducibility.

Analysis of Extracted Protein from Rat Brain. To further improve the identification capacity of such an integrated platform, a nanoHPLC based integrated platform was utilized, as shown in Figure 2b, and applied for the analysis of proteins (10 μg) extracted from rat brain. The results were compared with those obtained by the offline counterpart and MudPIT approach.

The base peak chromatograms obtained by an offline method, traditional MudPIT approach, and integrated approach were shown in Figure S-3 in the Supporting Information. Compared to the offline counterpart, it could be seen from Table 1 that not only more unique peptides (4404 vs 3942) and protein groups (1420 vs 1250) were obtained by integrated platform, but also the total analysis time, including sample preparation, separation, and identification, was greatly shortened. This might be caused by the high efficiency of on-column digestion and online organic modifier removal, and reduced sample loss by the integrated platform. Also, by comparison with traditional MudPIT approach, although the identified unique peptides and protein groups by integrated platform were slightly lower, which might be attributed to that the resolution of SCX for peptide fractionation was superior to that of RPLC for protein fractionation when stepwise gradient was applied, it was noteworthy that the analysis time of single run including sample pretreatment, digestion, separation, and detection was shortened to half of that taken by the MudPIT approach (19.5 vs 41.5 h).

Table 1. Analysis of Extracted Proteins from Rat Brain by Three Different Approach in Replicated Runs

	offline method	MudPIT approach	integrated approach
[a]separation time (h)	19.5	19.5	19.5
[b]sample pretreatment time (h)	22	22	
num of unique peptides	3942	5460	4404
[c]total num of protein groups	1250	1600	1420
[d]total num of protein groups	676	933	757

[a]The total separation time of single run includes protein sepration, peptide SCX fractionation, peptide desalting, peptide separation, and MS detection. [b]The total sample pretreatment time includes organic modifier removal and protein digestion. [c]The number of identified protein groups with minimum one peptide matching. [d]The number of identified protein groups with minimum two peptides matching.

To further improve reliability of identified proteins, protein groups matched with more than two distinct peptides were also summarized. As shown in Table 1, a total of 676, 933, and 757 protein groups matched with more than two distinct peptides were identified by an offline method, MudPIT approach, and integrated platform, respectively. Among which, 477 proteins were commonly identified by three different approaches, accounting for 70%, 51%, and 63% of identified proteins, and 88, 293, and 110 protein groups were exclusively identified, indicating that more comprehensive proteomic profiling might be obtained with different approaches.

Among these 757 proteins identified by integrated platform, it could be seen from Figure 4 that 11 (1.45%) proteins present with M_w less than 10 kDa, and 65 (8.58%) proteins, with M_w more than 100 kDa. The smallest and largest proteins were, respectively, of M_w 6.8 and 550 kDa. With the consideration of p*I*, 757 proteins were distributed cross the p*I* range from 3 to 12, among which 375 (49.6%) and 9 (1.18%) proteins were distributed between p*I* 3 and 5 and over 10, and 14 proteins were distributed between p*I* 7 and 8, demonstrating the capacity for the identification of various proteins by our developed integrated platform. All these results demonstrate that by such an integrated platform, high efficiency, high throughput, and automation for proteome profiling could be achieved.

CONCLUSION

A novel integrated platform for proteome analysis with the combination of protein separation by μRPLC, online removal of organic modifier and pH adjustment by an HFMI, online digestion by an IMER, and peptide separation and protein identification by μRPLC or nano-RPLC-ESI/MS/MS was established. By comparison with its offline counterpart, the advantages of reduced sample loss, good reproducibility, high throughput, and ease of automation were demonstrated in analysis of a three-protein mixture. Furthermore, such an integrated platform was applied for the analysis of extracted proteins from rat brain, by comparison with the offline

dx.doi.org/10.1021/ac3006796 | *Anal. Chem.* 2012, 84, 5124−5132

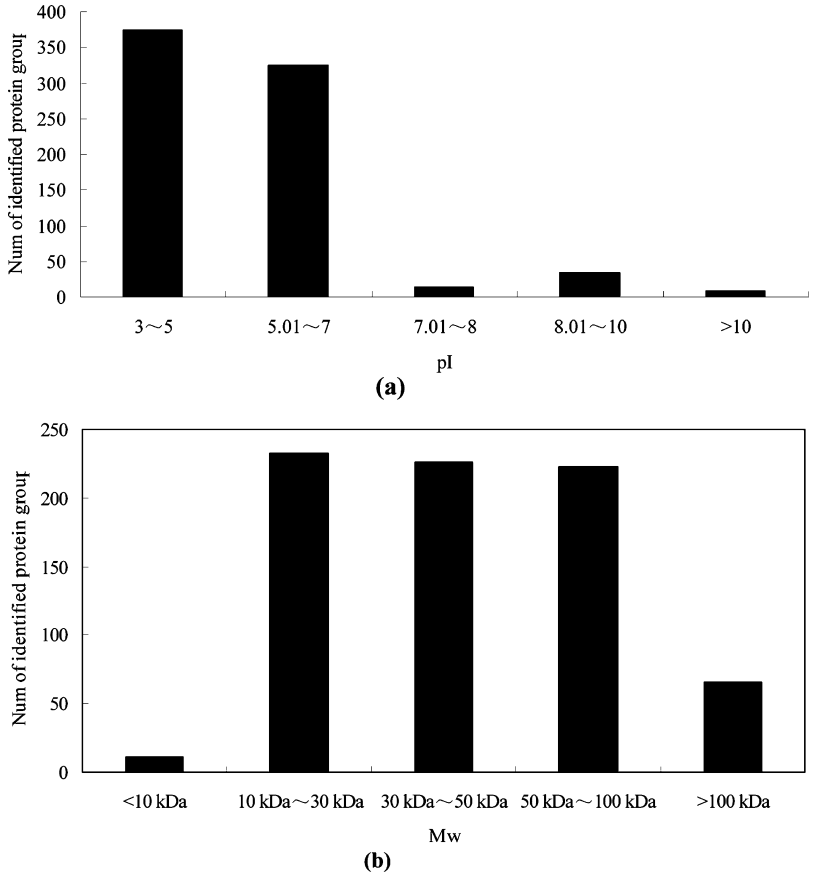


Figure 4. Isoelectric point (a) and molecular weight (b) distribution of rat brain protein groups identified by μRPLC-HFMI -IMER-nano-RPLC-ESI-MS/MS platform, with at least two distinct peptides matching.

counterpart and traditional MudPIT approach, a similar number of protein groups could be identified by integrated platform within less than half of the analysis time. All these results demonstrated that such an integrated platform might be an attractive alternative for high throughput and automatic proteome profiling.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Figures S-1−S-3 and Table S-1 and data files. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

Financial support from National Basic Research Program of China (2012CB910600), National Nature Science Foundation (20935004, 21027002 and 21105099), Creative Research Group Project by NSFC (21021004), Analytical Method Innovation Program of the Ministry of Science and Technology (2010IM030500), and Doctoral Fund of Dalian Institute of Chemical Physics (S201104) is gratefully acknowledged. We also thank Filippo Bedani (University of Amsterdam & Dalian Institute of Chemical physics) for the fruitful discussion. The first two authors contributed to this article equally.

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