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*Technical Notes*

# Online Integration of Multiple Sample Pretreatment Steps Involving Denaturation, Reduction, and Digestion with Microflow Reversed-Phase Liquid Chromatography−Electrospray Ionization Tandem Mass Spectrometry for High-Throughput Proteome Profiling

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**A facile integrated platform for proteome profiling was established, in which native proteins were online denatured and reduced within a heater, digested with an immobilized trypsin microreactor, and analyzed by microflow reversed-phase liquid chromatography with electrospray ionization tandem mass spectrometry (μRPLC-ESI-MS/MS). In comparison to the traditional off-line urea denaturation protocol, even more unique peptides were obtained by online heating in triplicate (14 ± 2 vs 11 ± 2 for myoglobin and 16 vs 12 ± 1 for BSA) within a significantly shortened pretreatment time of ∼3.5 min (including 1 min of thermal denaturation and reduction and ∼2.5 min of microreactor digestion). Moreover, proteins with concentrations ranging from 50 ng/mL (∼6 fmol) to 1 mg/mL (∼120 pmol) were positively identified by the online system. Such a platform was further successfully applied for analyzing the soluble fraction of mouse liver extract. Of all the 367 proteins identified from samples pretreated by the urea protocol and online heating, ∼40% were overlapped, showing the partial complementation of both approaches. All these results demonstrate that the online integrated platform is of great promise for high-throughput proteome profiling and improved identification capacity for low-abundance proteins with a minute sample amount.**

In the past decade, technology-driven proteomics has seen rapid development,[1] and mass spectrometry (MS) has evolved into an indispensable tool for in-depth proteome research.[2,3] However, a large-scale MS-based proteomic study requires high-throughput sample preparation techniques.[4] With the advances in multidimensional liquid chromatography techniques, the bottom-up strategy is becoming one of the most popular approaches for proteome profiling, by which proteins are in sequence denatured, reduced, alkylated, digested, and cleaned up prior to peptide separation with reversed-phase liquid chromatography (RPLC) and protein identification with electrospray ionization tandem MS (ESI-MS/MS).[4,5] Such a multistep sample pretreatment procedure might not only lead to sample loss or contamination but also is extremely tedious and time-consuming, not compatible with high-throughput analysis of proteomes.

Denaturation is a prerequisite step to ensure efficient protein digestion by changing the structure of proteins via destroying hydrogen bond, salt bridge, hydrophobic interaction, and van der Waals forces.[6] Generally, proteins can be denatured by either chemical or physical methods. For chemical denaturation, strong acid/base, urea, guanidine-HCl, organic solvents,[7] sodium dodecyl sulfate (SDS), acid-labile surfactants, and other detergents[8] have been applied. Although among them, urea and guanidine-HCl are commonly used, proteins denatured by high concentration salts

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might be refolded during dilution or desalting prior to protolytic digestion.[6] Such a problem could be avoided by denaturation with physical means, including heat,[9,10] pressure,[11] and microwave.[12] Together with denaturation, protein reduction is often required to facilitate digestion and identification, especially for proteins with multiple disulfide bonds in inter/intra polypeptide chains. However, in most cases, protein denaturation and reduction are performed off-line, resulting in extended handling time, potential sample loss, and difficulty in automation.

Protein digestion is another rate-limiting step for high-throughput proteome analysis. The typical in-solution digestion suffers from several drawbacks such as long incubation time (typically >5 h) and low digestion yields.[4] Recently ultrasound,[13] microwave,[12] infrared,[14] and alternating electric fields[15] have been employed to accelerate in-solution protein digestion. However, enzymatic activity might be simultaneously decreased under harsh conditions, and the autodigestion of enzymes might interfere with protein identification. As a promising alternative, on-column protein digestion with immobilized enzyme microreactors (IMERs) has attracted much attention in recent years.[16−18] To prepare such microreactors, enzymes are covalently bonded, trapped, or physically adsorbed onto different supports, such as particles or beads,[19,20] membranes,[21] plates,[22] the inner wall of fused silica capillaries,[23,24] and various kinds of monolithic materials.[25−31] Among them, monolith-based IMERs have great advantages such as excellent permeability and high enzymatic activity, resulting in substantially reduced digestion time for proteins.

Recently, several attempts have been made to achieve high-throughput proteome analysis by the partial or complete online integration of sample preparation, separation, and identification. Li et al.[32] integrated protein capture, sample cleanup, and digestion in a single stage with a hydrophobic microcolumn. After 30 min of sample loading and washing, 30 min of digestion, and 50 min of separation and identification, the obtained sequence coverage for cytochrome *c* and myoglobin were, respectively, 82% and 70%. Later, Figeys et al.[33] developed a microdevice termed as the proteomic reactor, by which protein adsorption, reduction, alkylation, digestion, and elution were performed on SCX resin, and 205 unique proteins were identified from 10 $\mu$g of mouse P19 cell lysate by nanoRPLC−ESI-MS/MS. Although it could be regarded as a platform integrated with multiple processing steps, samples might be lost due to the nonspecific adsorption on supports, and the whole pretreatment procedure took ~3 h.

Herein with the combination of online protein denaturation and reduction via heating, proteolytic digestion by IMER,[31] and peptide separation and protein identification by $\mu$RPLC−ESI-MS/MS, a fully integrated high-throughput platform was established and applied to the analysis of both standard proteins and the soluble fraction of mouse liver extract. Our results demonstrate that such an online platform will probably open a route for high-throughput proteome analysis and improved identification of low-abundance proteins.

## EXPERIMENTAL SECTION

**Materials and Chemicals.** Fused-silica capillaries (250 $\mu$m i.d. × 375 $\mu$m o.d.) were purchased from Sino Optical Fiber Factory (Handan, China). A precise syringe pump was obtained from Baoding Longer Pump Company (Baoding, China). A C8 trap column was ordered from Michrom Bioresources Inc. (Auburn, CA). Sinochrom ODS-AP particles (5 $\mu$m, 300 Å), a temperature displayer, and two stainless steel sample loops (0.005 in. i.d. × 20 cm length; 0.012 in. i.d. × 25 cm length) were obtained from Dalian Elite Analytical Instrument Co., Ltd. (Dalian, China). All PEEK tubing and stainless steel unions were purchased from Upchurch (Oak Harbor, WA). A sensitive platinum thermocouple (with an accuracy of ±0.5 °C in the range from −10 to 150 °C), heat-conductive glass fiber, and heating wires (20 Ω/m) were ordered from Haibo Mechanics and Electronics Service Center (Dalian, China). A multi-output transformer was supplied by Andeli Group Co., Ltd. (Wenzhou, China). Tetraethoxysilane (TEOS, 95%) and 3-aminopropyltriethoxy-silane (APTES, 99%) were obtained from Acros Organics (Geel, Belgium). Cetyltrimethyl ammonium bromide (CTAB) was supplied by Beijing Chemical Reagent Company (Beijing, China). TPCK-treated trypsin (bovine pancreas), myoglobin (equine skeletal muscle), cytochrome *c* (horse heart), and bovine serum albumin (BSA) were ordered from Sigma (St. Louis, MO). Dithioerythritol (DTT), iodoacetic acid (IAA), and phenylmethanesulfonyl fluoride (PMSF) were purchased from Amresco Inc. (Solon, OH). Tissue Tearor was obtained from Biospec Products (Bartlesville, OK). A Bradford protein assay kit was


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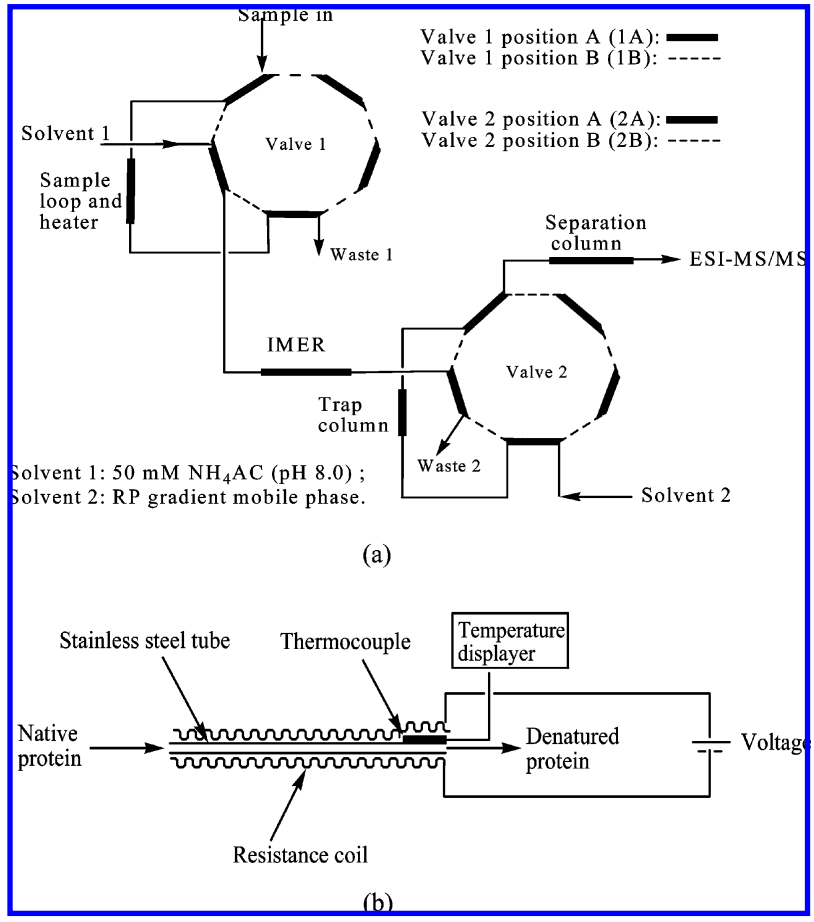


**Figure 1.** Scheme of the online system integrating protein denaturation, reduction, and digestion with μRPLC−ESI-MS/MS (a) and the enlarged view of the online heater (b).

ordered from Bio-Rad (Hercules, CA). Organic solvents were all of HPLC grade. Water was purified by a Milli-Q system (Millipore, Molsheim, France).

**Sample Preparation.** The traditional urea denaturation protocol was the same as described previously.[31] In brief, proteins were dissolved in 50 mM $CH_3COONH_4$ (pH 8.0) containing 8 M urea and then reduced in 10 mM DTT for 1 h at 56 °C. When cooled to room temperature, cysteines in proteins were alkylated by 20 mM IAA for 30 min at 37 °C in the dark, followed by the dilution with 50 mM $CH_3COONH_4$ (pH 8.0) to the desired concentration. Protein samples were prepared simply by dissolving in 50 mM $CH_3COONH_4$ (pH 8.0) containing 10 mM DTT without (for 0.1 mg/mL myoglobin or BSA) or with 1 M urea (for 1 mg/mL myoglobin or BSA) for online heating.

The mouse liver tissue was cleaned with Milli-Q water to remove possible contaminants, cut into small pieces, and homogenized with 1 mM PMSF solution using Tissue Tearor in ice bath. The homogenate was centrifuged at 12 000*g* for 40 min at 4 °C, and the supernatant (defined as soluble fraction) was collected. Also, the concentration of proteins was measured as ~2 mg/mL with a Bradford assay kit using BSA as a standard.

**System Setup.** The integrated platform for high-throughput proteome analysis is shown in Figure 1a, which consisted of a stainless steel sample loop with specially designed heater for protein denaturation and reduction, an IMER for digestion, a C8 trap column for peptide trapping, a GM4 μHPLC system (Michrom Bioresources Inc., Auburn, CA) for peptide separation, and an LCQ$^{DUO}$ quadrupole ion trap mass spectrometer (Thermo Fisher, San Jose, CA) for protein identification.

**Online Heater.** Schematic diagram of the heating device is illustrated in Figure 1b. Two stainless steel sample loops, respectively, with 0.005 in. i.d. × 20 cm length (~2.5 μL for standard proteins) and 0.012 in. i.d. × 25 cm length (~18.3 μL for complex samples) were designed as sample containers for online thermal denaturation and reduction. A sensitive platinum thermocouple connected with a temperature displayer was fixed at the outer wall of the sample loop. After prewrapped with a layer of heat-conductive glass fiber, the sample loop was tightly wrapped with heating wires, followed by three layers of glass fiber for heat insulation. A multi-output transformer was employed to convert the commercial alternating current (ac, 220 V, 50 Hz) into lower ac voltages for heating the sample loop to the desired temperature.

**Preparation of Organic−Inorganic Hybrid Silica Monolith-Based Microreactor.** The monolith-based immobilized trypsin microreactor was prepared according to our previous procedure,[31] with slight modifications. In brief, the capillary with inner diameter of 250 μm was filled with polymerization solution containing TEOS (112 μL), APTES (118 μL), anhydrous ethanol (215 μL), cetyltrimethyl ammonium bromide (8 mg), and water (32 μL), and then the polymerization was performed at 40 °C for 24 h to form organic−inorganic hybrid monolith. Subsequently, the monolithic support was activated by flushing a solution of 10% (v/v) 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) (v/v), respectively, for 4 h, the microreactor was cut into desired lengths, filled with 0.02% (w/v) $NaN_3$ solution, and stored at 4 °C before use.

For off-line evaluation of the IMER, proteins were pushed through the microreactor by a precise syringe pump and the resulting products were manually collected and analyzed by μRPLC−ESI-MS/MS. For online digestion, a flow rate of 1 μL/min was used to deliver denatured proteins to the microreactor at room temperature, and the digests were trapped on a C8 column.

**μRPLC−ESI-MS/MS and Data Analysis.** After valve switching, peptides adsorbed on a C8 trapping column were eluted into a homemade C18 microcolumn (300 μm i.d. × 15 cm) for separation. The mobile phase A was composed of 2% (v/v) ACN with 0.1% (v/v) formic acid, and mobile phase B was composed of 98% (v/v) ACN with 0.1% (v/v) formic acid. The flow rate was 5 μL/min by splitting. The first 10 min of elution with 0% B was utilized for desalting. Then, gradients with 60 and 170 min were applied to separate the digests of standard proteins and complex samples, respectively. ESI-MS/MS experiments were performed in the positive ion mode with a 3.0 kV applied voltage. The temperature of the heated capillary was set to 150 °C. Xcalibur software (version 1.4) was used to control the whole system and record total ion current chromatograms and mass spectra covering the *m/z* range from 400 to 2000. A full scan MS was followed by two (for standard proteins) or three (for complex samples) data dependent MS/MS events. The normalized collision energy for MS/MS scanning was 35%. Besides the manual inspection of mass spectra, data were also submitted to the SEQUEST algorism (Bioworks Software 3.1) for further analysis. For database searching, main parameters were set as follows: enzyme, trypsin; missed cleavages, 2; precursor-ion mass tolerance, 2 Da; fragment-ion mass tolerance, 1 Da; static modification, Cys (+57) for the samples pretreated with the urea protocol; dynamic modification, Lys or Arg (+44) for possible carbamylation for thermal denatured proteins containing 1 M urea. All databases were newly downloaded from the Web site of National Center for Biotechnology Information (www.ncbi.nih.gov, Bethesda, MD). The digests of myoglobin and BSA were searched against *equine*.fasta and *bovine*.fasta, respectively, and the results were filtered by the Xcorr(+1, 2, 3) = 1.90, 2.20, 3.75 for positive identification. The *ipi.MOUSE.v3.19*.fasta and its reverse database were separately searched for digests of the soluble fraction from mouse liver extract. Stringent criteria of Xcorr(+1, 2, 3) = 1.90, 2.20, 3.75 and Cn ≥ 0.3 were set to control the false discovery rates (FDRs) less than 5%. Experiments were conducted in triplicate.

## RESULTS AND DISCUSSION

The goal of this research is to provide an efficient and reliable online platform integrated multiple pretreatment steps, including protein denaturation and reduction by heating and digestion by IMER, with peptide separation and identification by μRPLC−ESI-MS/MS for high-throughput proteome analysis.

**Thermal Denaturation.** Thermal denaturation prior to enzymatic digestion was first used in the field of food nutrition.[34] Early studies revealed that temperature increase might lead to not only the weakening of noncovalent forces in protein macromolecules but also the exposure of hidden hydrophobic groups in molecular core to solvents.[35] However, oligomers of denatured proteins might be formed by heating, resulting in protein aggregation and thus decreased accessibility toward proteases. To solve this problem, several factors that affect protein aggregation, including protein concentration and, most importantly, the physical and chemical properties of proteins, were investigated.

Three typical proteins, BSA, myoglobin, and cytochrome *c*, respectively, representing acidic, natural, and basic ones, were dissolved in 50 mM $CH_3COONH_4$ (pH 8.0) with or without 1 M urea added and then heated in a water bath at 90 °C for 10 min (as shown in the Supporting Information, SI-Table 1). No apparent aggregates of BSA and cytochrome *c* were found even with the concentration up to 1 mg/mL with or without the addition of urea. In contrast, obvious aggregates of 1 mg/mL myoglobin were observed without urea added, while only a few were formed with the presence of 1 M urea. These results indicate that, to some extent, the addition of a certain amount of additives (i.e., 1 M urea) can inhibit the aggregation of high concentration proteins upon heating, similar to what Betenbaugh et al. observed.[36] Therefore, 1 M urea was added in the buffer for the analysis of proteins with the concentration of 1 mg/mL.

**Evaluation of IMER.** In recent work, we have developed an immobilized trypsin microreactor in a 100 μm i.d. capillary with organic−inorganic hybrid silica monolith as the matrix, prepared by the sol−gel method with TEOS and APTES as precursors with the presence of CTAB.[31] Since the optimum flow rate for protein digestion was 300 nL/min, it was not suitable for the purposed online microflow analytical platform herein. Therefore, a microreactor with a large bore of 250 μm i.d. was prepared under the conditions as previously reported.[31]

A standard protein myoglobin, with obvious aggregation susceptivity and digestion resistance as mentioned above, was used to evaluate the performance of IMER. When 0.1 mg/mL myoglobin pretreated with the urea protocol was pushed through the microreactor, the digests were collected and then subjected to μRPLC−ESI-MS/MS. With a flow rate of 1 μL/min, corresponding to a residence time of ~2.5 min in the microreactor, the sequence coverage of 79 ± 0.6% for 0.1 mg/mL myoglobin (~10 pmol) was obtained (SI-Figure 1a in the Supporting Information). Also, no significant decrease on the coverage was observed with the flow rate up to 5 μL/min. To enhance the accessibility of proteins and immobilized enzyme, a relatively lower flow rate of 1 μL/min was used in the following experiments.

Because of the benefit of increased salt concentration for the inhibition of protein aggregation upon heating, the effect of salt concentration on the enzymatic activity of IMER was studied. With $CH_3COONH_4$ concentrations ranging from 50 to 1000 mM, the variance of sequence coverage of 0.1 mg/mL myoglobin was

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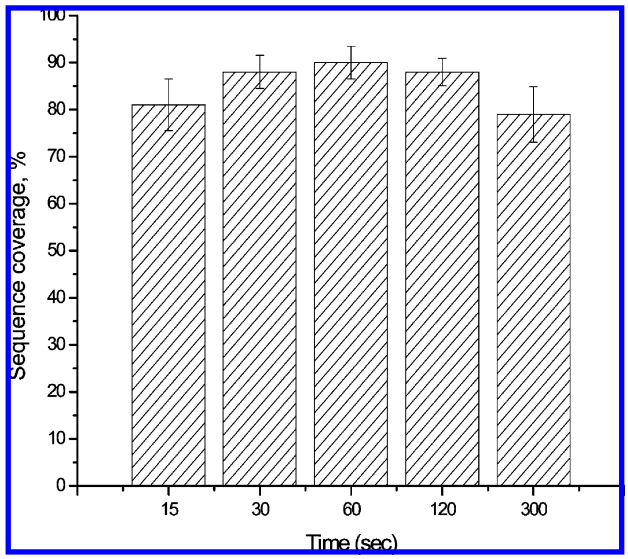


**Figure 2.** Effect of online heating time on the sequence coverage of myoglobin. Conditions: sample injection, 2.5 $\mu$L of 0.1 mg/mL myoglobin with 10 mM DTT; thermal denaturation, 90 °C; microreactor, 250 $\mu$m i.d. × 5 cm, 25 °C; other conditions are as in the text.

not obvious (SI-Figure 1b in the Supporting Information). The excellent salt-resistance of the microreactor might be ascribed to the short digestion time, during which the accessibility between internal peptide bonds and trypsin active sites was not significantly changed, in accordance with the previous report.[27] Therefore, the addition of 1 M urea in high concentration proteins could hardly affect the activity of IMER.

**Analysis of Standard Proteins by Online System.** As illustrated in Figure 1a, the sample loop, serving as an online heater as well, was heated to the desired temperature before sample injection. After a certain time of incubation in the loop, the denatured proteins were pumped through the microreactor via a 30 cm long PEEK tube, by which the temperature was cooled down to room temperature, enabling online proteolytic digestion.

Our preliminary study showed that 0.1 mg/mL myoglobin was not denatured completely with temperatures below 90 °C for 2 min, as evidenced by the existence of intact protein in chromatograms of the resulting digests (data not shown). Therefore, 90 °C was chosen for online heating.

With the integrated platform, the effect of online heating time on the identification of myoglobin was studied. As shown in Figure 2, the sequence coverage of 81 ± 5.5% for 0.1 mg/mL myoglobin (~12 pmol) was achieved even with a short processing time of 15 s, indicating the excellent thermal denaturation and microreactor digestion efficacy of the online system. With the heating time increased to 1 min, relatively higher sequence coverage of 90 ± 3.5% was obtained. However, with the further increase of heating time to 5 min, the yielding sequence coverage was decreased to 79 ± 5.9%, which might be caused by the fact that the aggregation of denatured proteins over time might hinder proteolytic digestion.[36] Thus the heating time of 1 min was used for standard proteins and extended to 2 min for complex samples.

For comparison, 0.1 mg/mL myoglobin pretreated by the traditional urea denaturation protocol was also subjected to online digestion followed by $\mu$RPLC−ESI-MS/MS under the same conditions (without heating for the sample loop). As shown in Table 1, with triplicate analyses, more unique peptides and higher sequence coverage were yielded by thermal denaturation than those obtained by the urea protocol (14 ± 2 vs 11 ± 2 peptides and 90 ± 3.5% vs 80 ± 1.5% coverage; with details shown in SI-Table 2 in the Supporting Information), and the sample pretreatment time was substantially shortened from 1.5 h to 1 min, indicating enhanced denaturation performance with online heating.

**Table 1. Results of Identified Proteins Pretreated by Online Heating and the Urea Protocol with Three Consecutive Runs**

| | myoglobin (~12 pmol) | | BSA (~3 pmol) | |
|---|---|---|---|---|
| pretreatment mode | online heating | the urea protocol | online heating | the urea protocol |
| time | 1 min | 1.5 h | 1 min | 1.5 h |
| unique peptides | 14 ± 2 | 11 ± 2 | 16 | 12 ± 1 |
| sequence coverage, % | 90 ± 3.5 | 80 ± 1.5 | 34 ± 2.1 | 28 ± 5.5 |

The performance of the online system for a high concentration of protein was evaluated with the analysis of 1 mg/mL myoglobin (~120 pmol; containing 10 mM DTT and 1 M urea). A total of 15 ± 1 unique peptides with the sequence coverage of 80 ± 4.0% were obtained, demonstrating that protein samples with the concentration up to 1 mg/mL were effectively unfolded and digested. In addition, a low concentration of myoglobin down to 50 ng/mL (~6 fmol; containing 10 mM DTT) was successfully identified as well, and 3 ± 1 matched peptides with the coverage of 24 ± 3.8% were achieved. These results imply that the applicable protein concentration range for our integrated platform is from 50 ng/mL to 1 mg/mL.

Moreover, another globular protein BSA with a large molecular weight of 69 kDa and multiple disulfide bonds was analyzed. As shown in Figure 3, in comparison to the urea protocol, slightly higher ion signals were recorded and more unique peptides with higher sequence coverage for 0.1 mg/mL BSA (~3 pmol) were obtained by online heating within 1 min (16 vs 12 ± 1 peptides and 34 ± 2.1% vs 28 ± 5.5% coverage; with details shown in SI-Table 3 in the Supporting Information), demonstrating the capacity for the analysis of various proteins with the online system.

For further comparison, off-line protein thermal denaturation at 90 °C for 20 min followed by in-solution digestion at 37 °C for 3 h (trypsin/protein = 1/40 (w/w)), as proposed by Russell et al.,[9] was performed, and the digests were subjected to $\mu$RPLC-ESI-MS/MS with experimental conditions the same as those for our online platform. A total of 9 ± 1 and 5 ± 1 peptides, corresponding to the coverage of 69 ± 7.5% and 9 ± 2.1%, respectively, were achieved for 0.1 mg/mL myoglobin (~12 pmol) and BSA (~3 pmol), less than those obtained by the integrated system, which might be ascribed to the low digestion performance resulting from both the presence of disulfide bonds (without DTT added) and the relatively lower trypsin to protein ratio by in-solution digestion. Moreover, when 10 $\mu$g/mL (~1 pmol) myoglobin was analyzed with the off-line method, 1 peptide with 9% coverage was obtained. Therefore, relatively higher concentrated samples (≥10 $\mu$g/mL) have to be applied for positive identification with the off-line method, while only 50 ng/mL (~6 fmol) protein is needed for the online platform. Besides less sample loss, the higher trypsin concentration in the confined space of the microreactor and thus enhanced digestion efficacy for low

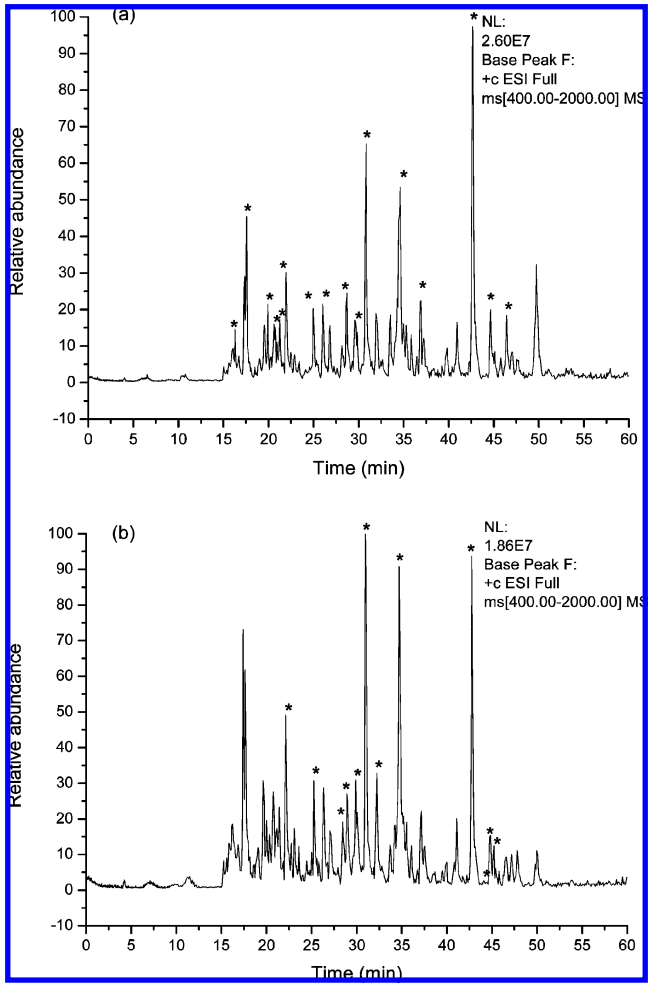


**Figure 3.** Base peak chromatograms of the digests of BSA pretreated by online heating (a) and the urea protocol (b). Conditions: sample injection, 2.5 $\mu$L of 0.1 mg/mL BSA with 10 mM DTT; thermal denaturation, 90 °C, 1 min; microreactor, 250 $\mu$m i.d. × 5 cm, 25 °C; other conditions are as in the text. The peaks labeled with "*" represent matched peptides of BSA.

concentration proteins might be the main cause for this significant discrepancy. Also, it is anticipated that the online system would be superior for the analysis of trace proteome samples and for the discovery of important functional proteins (e.g., growth factors and cytokines) even with low abundance in tissues and cell lines.[1]

It should be pointed out that, besides efficient denaturation and digestion of proteins within a few minutes, the integrated online platform features other advantages. (1) With the addition of a certain amount of reducing reagents (i.e., 10 mM DTT) in protein solution, chemical reduction of disulfide bonds can be simultaneously performed with thermal denaturation within 1 min, which might be contributed by increased reaction rates at elevated temperature. (2) No additional cysteine alkylation is required. Since the denatured proteins are promptly digested with the microreactor and the digests are readily analyzed by $\mu$RPLC−ESI-MS/MS and each mobile phase is degassed by nitrogen as well, oxidation can not occur on the thiol groups of proteins and/or their digests during the whole online analytical process. (3) Because of the irreversible denaturation upon heating, denatured proteins can not be refolded during dilution, rendering the disrupted structures more accessibility for proteolytic digestion. (4) Different from the traditional in-solution digestion, in which a certain amount of acid (i.e., 1% formic acid) has to be added to terminate the enzymatic reaction,[4] real-time acidification is achieved herein by the mobile phase of $\mu$RPLC which contains 0.1% formic acid. (5) Desalting of peptides can be performed online prior to MS detection. Although 1 M urea and 10 mM DTT are added in high concentration samples (i.e., 1 mg/mL), they can be desalted during two periods. When the digests are retained by the C8 trapping column, a large volume of 50 mM $CH_3COONH_4$ can be used for flushing out most of the nonvolatile salts. Also, the possible remaining salts can be further removed during the 10 min rinse with 0% mobile phase B before gradient elution of $\mu$RPLC started. (6) The whole analysis procedure can be controlled automatically, and extra manual operation and possible sample loss can be avoided.

**Complex Sample Analysis.** To evaluate the applicability of our integrated platform for complex samples, the soluble fraction extracted from mouse liver was analyzed. The native proteins (~1 mg/mL, containing 10 mM DTT and 1 M urea) were injected, online denatured and reduced by heating at 90 °C for 2 min, digested with a 10 cm long microreactor (corresponding to a residence time of ~5 min), and analyzed by $\mu$RPLC−ESI-MS/MS with a relatively longer gradient elution time of ~3 h. As a control, another 1 mg/mL protein sample pretreated with the traditional urea protocol was analyzed as well (as illustrated in SI-Figure 2a,b in the Supporting Information). After database searching with FDRs less than 5%, 260 ± 43 unique peptides were matched and 137 ± 16 proteins were identified by online heating, while 357 ± 26 unique peptides and 172 ± 10 proteins were yielded by the urea protocol. In contrast to ~1.5 h required for the urea protocol, efficient denaturation and reduction were achieved for complex samples by the online system within 2 min. Different mechanisms for protein denaturation, resulting in different structures of unfolded proteins[37] and different accessibility to the immobilized enzyme, might attribute to the slightly less number of identified proteins with online heating.

To improve the accuracy and number of identified proteins, repeated analysis has been regarded as a good solution.[38] After three consecutive runs, the number of identified nonredundant proteins was increased by 27% and 46% for samples denatured, respectively, by the urea protocol and online heating (Figure 4). With the combination of all proteins identified by both pretreatment approaches in triplicate, in total 367 proteins were identified (269 proteins from the urea protocol and 244 proteins from online heating). Among which, only 146 proteins (~40%) overlapped, indicating the partial complementation of two such methods for protein pretreatment. Therefore, more comprehensive proteomic profiling might be obtained with parallel sample preparation by the urea protocol and heating. Moreover, in comparison to the overlap ratio of proteins identified with $\mu$RPLC−ESI-MS/MS (>50%) for one sample in triplicate,[38] less protein overlap ratios, ~36% for pretreatment with the urea protocol and ~25% with online heating followed by digestion were achieved herein, which might be ascribed to the different denaturation methods and proteolytic digestion variance.

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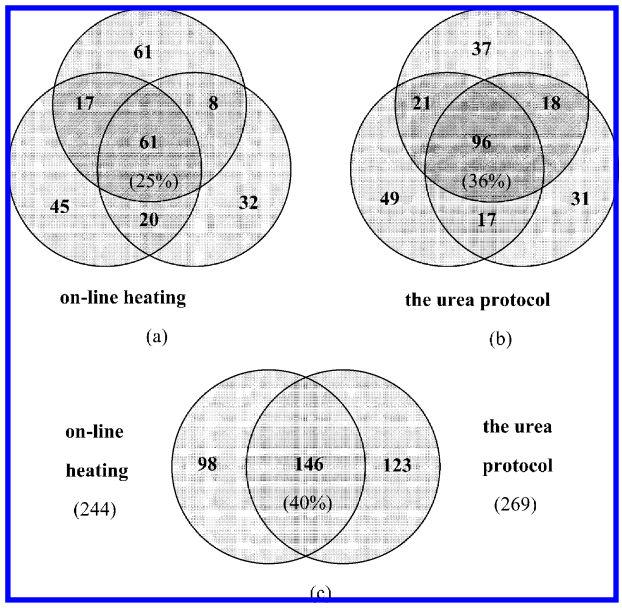


**Figure 4.** Venn diagram of the identified proteins from the soluble fraction of mouse liver extract with two pretreatment approaches ($n$ = 3). Conditions: sample injection, 18.3 $\mu$L of 1 mg/mL extracted proteins; thermal denaturation, 90 °C, 2 min; microreactor, 250 $\mu$m i.d. × 10 cm, 25 °C; flow rate, 1 $\mu$L/min; other conditions are as in the text.

It should be noted that, according to the PSORT Prediction Software (http://psort.ims.u-tokyo.edu.jp), about 5% of the proteins (13/244) were identified as membrane proteins from the soluble fraction with the online platform. It seems that our purposed setup is applicable to handle some hydrophobic proteins besides the soluble ones.

For further comparison, with a slightly adapted procedure from Russell et al.,[9] the same fraction (containing 10 mM DTT and 1 M urea) of mouse liver extract was off-line pretreated by heating at 90 °C for 20 min followed by in-solution digestion at 37 °C for an extended time of 24 h, and the digests were subjected to $\mu$RPLC−ESI-MS/MS with the same experimental conditions as those for the online platform. Even though a longer sample pretreatment time of ~24.5 h was used, only 117 ± 4 unique peptides and 75 ± 4 proteins (FDRs < 5%; $n$ = 3) were recognized, which might be caused by the lesser efficacy of in-solution digestion for low concentrated proteins, difficulty in peptide assignment due to the possible relinkage of free thiol groups exposed to the air during digestion, and possible sample loss. Moreover, almost 80% of the identified proteins were covered in the data set obtained by our integrated system, further demonstrating the superiority of the online platform for protein profiling even for complex samples.

It is also noteworthy that, although the number of proteins identified from the soluble fraction with our online protocol is less than that in the largest data set of the mouse liver whole proteome by Mann et al.,[39] from which subcellular fractionation, protein separation, urea denaturation, DTT reduction, IAA alkylation, in-gel/in-solution digestion, and nanoRPLC separation followed by identification with advanced MS instruments were employed, ~35% (86/244) of the proteins were exclusively identified with our online platform (SI-Table 4 in the Supporting Information). It is expected that a more complete protein list would be achieved by combining different sample processing protocols.

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## CONCLUSIONS

An integrated online platform involving denaturation, reduction, and digestion followed by separation and identification with $\mu$RPLC−ESI-MS/MS was established for high-throughput proteome profiling, by which the sample pretreatment time was obviously shortened from typically several hours or even 1 day to ~3.5−7 min, including 1−2 min of thermal denaturation and reduction and ~2.5−5 min of microreactor digestion. Furthermore, in comparison to the traditional off-line sample preparation method, cysteine alkylation, termination of digestion, and peptide desalting could be avoided and more importantly the whole procedure could be performed automatically without the risk of sample loss or contamination. In addition, the required minimum sample amount for protein identification could be decreased to the level of femtomoles. All these benefits enable high-throughput treatment and analysis of proteomes with the purposed online platform.

Besides serving as a fixed sample loop, the online heater could also be assembled at other places in the flow-through pipeline for denaturation and reduction, thus rendering such a sample processing unit much flexibility for online pretreatment. Further work on constructing more integrated platforms including protein separation, real-time denaturation and reduction with heating, fast digestion with IMER followed by 2-D nanoLC−ESI-MS/MS is underway in our lab for large-scale and in-depth proteome profiling.

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## NOTE ADDED AFTER ASAP PUBLICATION

This manuscript originally posted ASAP on July 7, 2009. Corrections were made to the Supporting Information and text was added to the Supporting Information paragraph, and this corrected version posted ASAP on July 10, 2009.

## SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. SI-Tables 1–3 and SI-Figures 1 and 2 are in the file ac900971w_si_001.pdf, and SI-Table 4 is in the file ac900971w_si_002.xls. This material is available free of charge via the Internet at http://pubs.acs.org.

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