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Porous silica enhanced proteolysis during Off-Gel separation for efficient protein identification

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ABSTRACT

An advanced approach is developed in this work for simultaneous on-line separation and digestion of proteins by combining the Off-Gel isoelectric focusing (IEF) and enzymatic nanoreactor enhanced proteolysis. The nanoreactor was prepared by preloading trypsin in amino-functionalized macroporous silica, and then directly added into Off-Gel wells. With the nanoreactor loaded Off-Gel device, effective digestion of proteins happened during IEF electrophoresis to generate directly fractionated tryptic peptides, which not only accelerated the experimental flow but also avoided sample loss, leading to a more comprehensive protein identification from complex biological samples. A successful identification of 3592 proteins was achieved from Hela cell line by using the approach followed with LC–MS/MS analysis, while only 1877 proteins were identified from the same sample when using standard in-solution proteolysis followed with Off-Gel electrophoresis and then LC–MS/MS analysis. Therefore, we have demonstrated that the approach can greatly simplify high-throughput proteomics and significantly improve protein identification.

1. Introduction

Proteomics has progressed tremendously over the last decade. In the current qualitative and quantitative proteome approaches, fractionation of complex samples has been recognized as one of the most important steps to ensure the high sensitivity and sequence coverage for protein identification [1,2]. Poor fractionation can lead to problems in ionization, spectra acquisition, and signal suppression of low-abundance species during mass spectrometry (MS) analysis. Various techniques have been developed to reduce the complexity of proteins and peptides extracted from biological samples [3–6], *e.g.* size exclusive chromatography (SEC), isoelectric-focusing (IEF) electrophoresis, strong-cation exchange (SCX), reversed-phased liquid chromatography (LC) and *etc.* Among the techniques, Off-Gel IEF electrophoresis (OGE) is widely used to fractionate proteins and peptides by their isoelectric points (*pI*), and to recover the purified compounds directly in solution [7–10]. Fractioned proteins or peptides are buffered by the immobilized pH gradient (IPG) gel used for separation, and can be further analyzed by the standard LC–MS/MS workflow.

Depending on the complexity of sample and desired resolution, a typical OGE takes 6 to 24 h. It can be used for the fractionation of proteins extracted from tissue or cells, and then the peptides digested from each protein fraction [11]. In coupling with capillary zone electrophoresis (CZE), the 2D separation can provide orthogonality comparable with reverse phase chromatography coupled to strong cation exchange chromatography (RP/SCX) [12]. However, a typical OGE-MS proteome strategy is a tedious process requiring the collection of fractionated proteins, adjusting the system pH of each fraction into preferable condition for trypsin digestion, offline overnight proteolysis, and peptide fractionation [11,13,14]. Sample handling between the steps can lead to sample loss. To circumvent the time and sample consuming procedures, it is very important for large-scale proteome research to develop novel approaches able to combine the separation of proteins/peptides and efficient proteolysis in a single step.

We have demonstrated in a previous study that macroporous silica loaded enzyme can be used for fast proteolysis under various

Abbreviations: MOWSE, MOlecular Weight SEarch; MALDI-TOF MS, Matrix assisted laser desorption/ionization time-of-flight mass spectrometry; LC–ESI–MS/MS, Liquid chromatography–electrospray ionization–tandem mass spectrometry; SEM, Scanning electron microscopy; OGE, Off-Gel electrophoresis; FTIR, Fourier transform infrared spectra

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conditions [15]. With theoretical predictions and experimental validations, it is demonstrated that the porous nanomaterials efficiently accelerate biochemical reactions because they can enrich reactants into their inner pores (enrichment effect), and confine reactions in nanoscale (nanoconfinement effect) [16,17]. Macroporous materials with the pore diameters of ~100 nm are currently proven as the perfect matrices to accelerate proteolysis rate, since they can provide optimum extraction and confinement to proteins (several to tens of nanometer in diameter) compared to mesoporous materials with smaller pores that give steric inhibition [15,18–20]. Furthermore, with inner surface modification, the local enviroment inside nanopores can be adjusted that is not influenced by the bulk buffer condition outside the pores. Therefore, an amino-functionalized macroporous silica foam (NH_2-MOSF) mediated digestion protocol was applied to a wide range of bulk buffer pH conditions [21], where the amino groups made the local condition appropriate to maintain the tryptic activity for ultra-efficient proteolysis.

In this study, trypsin preloaded NH_2-MOSF nanoreactor was directly added to OGE wells with pH from 3 to 10 to carry out online digestion during OGE separation. To date, it is the first strategy to realize proteolysis during OGE separation. By loading proteins in the OGE wells, fractionated peptides with appropriate *pI* could be directly recovered from the corresponding OGE wells at the end of experiments for further MS or LC–MS/MS analysis. The method provides a simple, fast and efficient OGE-MS based proteome flow to minimize sample loss, and to enhance protein identification. The concept was firstly demonstrated by using a standard protein mixture, and then applied for biological complex samples (Hela cell protein extracts). Successful identification of 3568 proteins from Hela cell line was achieved. In contrast, only 1851 proteins were identified from the same sample with the traditional OGE-MS method where in-solution proteolysis was performed prior to the OGE of peptides.

2. Materials and methods

2.1. Materials and reagents

Potassium chloride (KCl, 99.5%) was from AnalaR, Australia. Ethanol (99.5%) was from Asia Pacific Specialty, Australia. Alpha-cyano-4-hydroxycinnamic acid (CHCA, 99%), acetonitrile (ACN, 99.9%), and trifluoroacetic acid (TFA, 99.8%) were purchased from Merck. Triblock copolymer $EO_{20}PO_{70}EO_{20}$ (denoted P_{123}, where EO is poly(ethylene oxide) and PO is poly(propylene oxide)), ammonium bicarbonate (98%), dry toluene, 3-aminopropyltriethoxysilane (APTS, 97%), tetramethylorthosilicate (TMOS, 99%), Trypsin (from bovine pancreas), myoglobin (from horse heart), bovine serum albumin (BSA), beta-casein (from bovine milk), cytochrome C (cyt C, from horse heart), 4-(2-hydroxyerhyl) piperazine-1-erhaesulfonic acid (HEPES, 99.5%), dithiothreitol (DTT, 99.0%), and IAA (iodoacetamide, 98%) were all obtained from Sigma-Aldrich (Schnelldorf, Germany). All buffer and protein solutions were prepared with deionized water from a milli-Q system (Millipore, Bedford, MA, USA).

2.2. Synthesis and characterization of macroporous silica and the amino-functionalized materials

The macroporous silica foam (MOSF) used in this study was prepared according to the method previously reported [20]. The synthesis of MOSF was conducted at 35 °C in pH 5.0 NaAc–HAc buffer, using tetramethylorthosilicate as a silica source and P_{123} as a template agent. For standard modification, APTS was used as the coupling agent. MOSF materials were firstly dried and degassed at 110 °C for 6 h, and then dispersed in dry toluene (0.1 g MOSF in 30 g toluene). An excess of APTS (3 mL) was added in the MOSF toluene suspension under stirring and the mixture was stirred and refluxed at 110 °C for 24 h. The resulting solid was filtered and washed sequentially by toluene (200 mL), dichloromethane (200 mL), and ethanol (500 mL), respectively. The final NH_2-MOSF products were obtained after drying at 70 °C for 12 h.

A zeta-potential meter (Malvern Zetasizer Nano) was used to measure the zeta potential of the material in an ammonium bicarbonate (25 mM, pH 8) buffer at 25 °C (MOSF and NH_2-MOSF, 1 mg/mL each). Fourier transform infrared spectra were obtained with a FT-IR360 (Nicolet). Scanning electron microscopy (SEM) images were recorded on a Hitachi S-4800 microscope operating at 1 kV. The samples were coated with gold before SEM observation.

2.3. Adsorption of proteins or enzymes by NH_2-MOSF

Kinetic experiments to determine the amount of trypsin (molecular diameter ~3.8 nm, molecular mass ~23.8 kDa) adsorbed into NH_2-MOSF as a function of time were conducted by mixing 1 mL of 1.0 mg/mL^{-1} protein aqueous solution with 1 mg of NH_2-MOSF under stirring at 25 °C. The supernate was collected for UV/vis analysis to quantify the protein concentration after adsorption. The characteristic adsorption at 280 nm of protein was detected for quantification.

2.4. Digestion of standard myoglobin under different pH

In-solution digestion was performed according to the standard procedure [22] but with different buffer pH conditions. Myoglobin, previously denatured by heating at 100 °C for 5 min, was prepared (60 μg/mL) in buffer with pH ranging from 8 (25 mM ammonium bicarbonate) to 10 (adjusting ammonium bicarbonate with ammonia solution), and incubated at 37 °C with trypsin for 10 min with an enzyme/substrate ratio of 1:10 (w/w).

For NH_2-MOSF mediated digestion, the trypsin preloaded NH_2-MOSF (0.05 mg enzyme per 1 mg material) was directly dispersed in the above described protein solution (myoglobin 60 μg/mL) to reach a final NH_2-MOSF concentration of 0.12 mg/mL, and incubated at 37 °C for 10 min. The digestion products were analyzed directly by matrix-assisted laser desorption/ionization (MALDI) MS (Applied Biosystems, 5800 Proteomics Analyzer, Framingham, MA, USA). Loading of trypsin to NH_2-MOSF was performed by mixing 1 mL of 0.05 mg/mL^{-1} protein aqueous solution with 1 mg of NH_2-MOSF under stirring at 25 °C.

2.5. Proteolysis of protein mixture during Off-Gel electrophoresis

OGE separation was carried out with an Agilent 3100 OFFGEL fractionator (Agilent Technologies, Waldbronn, Germany). 18-cm immobilized pH gradient (IPG) strips (pH 4-7, Amersham Biosciences, Otelfingen, Switzerland) were used for experiments that allowed the collection of 18 fractions. 60 μL of protein mixture containing cytochrome C, myoglobin, BSA and beta-casein (93 μg/mL each, previously denatured by heating at 100 °C for 5 min) was loaded into each OGE well. Subsequently, 4 μL of the suspension of NH_2-MOSF (4 mg/mL) pre-loaded with trypsin (0.05 mg per 1 mg material) in water was added to each well. Finally, each OGE well was filled with deionized water up to a total volume of 150 μL. The OGE was carried out for 14 h, while the voltage and current were limited to 3.5 kV and 150 μA, respectively. To enhance the efficiency of protein digestion, OGE separation was performed at 35 °C for the first 2 h and then at 15 °C for the rest of the focusing period to avoid quick solvent evaporation. At the end of the fractionation, the fractionated peptide

solution in each well was collected and further analyzed by MALDI–MS (Bruker microflex LRF, Bremen, Germany).

2.6. MALDI–MS analysis

Alpha-cyano-4-hydroxycinnamic acid (CHCA, 5 mg/mL in 50% water, 49.9% acetonitrile and 0.1% trifluoroacetic acid) was used as MALDI matrix in all the experiments. All mass spectra were obtained in the positive reflectron mode. For the analysis of standard proteins without OGE, mass spectrometric data analysis was performed by peptide mass fingerprinting (PMF) methods with Mascot as search engine and Swiss-Prot 2014_09 (Mammalia) as database with one maximum missed cleavage site accepted and peptide mass tolerance of 80 ppm. For the analysis of standard protein mixture after OGE separation, online tools (FindPept, www.expasy.org) were used to match the observed peaks based on molecular mass, where only specific tryptic digests of the four standard proteins, cytochrome C (horse), myoglobin (horse), BSA (bovine), and beta-casein (bovine), were considered, with mass tolerance of $\pm$ 0.2 Da.

2.7. Preparation of whole cell lysate from Hela cells

Hela cells were grown in cell culture dishes until confluency. Cell layers were digested by trypsin and then rinsed with phosphate buffered saline (6.7 mM PO_4, HyClone, Thermo Scientific). Cell suspensions were centrifuged for 5 min at 1000*g* and the resulting pellets were resuspended in 10 mM HEPES buffer (pH 7.4,) and placed in ice bath for 30 min. After incubation, cells were burst by sonication for 20 times (2 s each time), and Triton X-100 (final concentration 1%) was added to solubilize proteins. Then, samples were ultrasonicated for 30 min at 4 °C. After centrifugation at 10,000*g* for 30 min at 4 °C, the protein-containing supernatant was collected for the following experiments.

The protein-containing supernatant was treated with DTT (final concentration 10 mM) and incubated at 37 °C for 45 min. Afterward, free thiol moieties were alkylated by adding IAA (final concentration 55 mM) for 45 min in darkness. Then, the protein extract was precipitated in 5 volume of ice-cold acetone under −20 °C overnight. After centrifugation at 14,000 rpm for 15 min, the pellet was washed and centrifuged a second time. The supernatant was decanted, and the remaining acetone was evaporated at room temperature. The dried protein pellet was resuspended with ammonium bicarbonate buffer (25 mM, pH 8), and deionizaed water respectively. Protein concentration was determined by Bradford assay as 1.5 mg/mL.

2.8. OGE-LC–MS/MS based analysis of Hela cell extract

OGE separation was carried out with the Agilent 3100 OFFGEL fractionator. 24-cm immobilized pH gradient (IPG) strips (pH 3–10 NL, Agilent Technologies) were used for all the experiments that allowed the collection of 24 fractions. 24 μL of protein mixture (1.5 mg/mL in deionized water) was loaded to each OGE well. Subsequently, 6 μL of NH_2-MOSF (4 mg/mL) preloaded with trypsin (0.05 mg per 1 mg materials) in water was added to each well to keep an enzyme/protein ratio of 1/30 (w/w) before electrophoresis. Finally, each OGE well was filled with the Off-Gel buffer (Agilent Technologies, Waldbronn, Germany) up to a total volume of 150 μL. The OGE was carried out for 25 h while the voltage and current were limited to 3.5 kV and 150 μA, respectively. The OGE separation was performed at 35 °C for the first 4 h and then at 15 °C for the rest of the OGE period.

For comparison, the same protein mixture (1.5 mg/mL, 576 μL in ammonium bicarbonate buffer) was digested at 37 °C with an enzyme/protein ratio of 1/30 (w/w) for 12 h in the buffer of 25 mM ammonium bicarbonate (pH 8), and then the proteolysis products were separated by OGE (25 h with the voltage and current limited to 3.5 kV and 150 μA, respectively, under 15 °C).

The fractionated peptide solution in each well was collected, desalted, dried, and further analyzed with LC–electrospray ionization (ESI)–MS/MS. LC was performed on a nano Acquity UPLC system (Waters Corporation, Milford, USA) connected to a LTQ Orbitrap XL mass spectrometer (Thermo Scientific, Bremen, Germany) equipped with an online nano-electrospray ion source (Thermo Scientific). Peptides from OGE were resuspended with 20 μl solvent A (5% acetonitrile, 0.1% formic acid in deionized water). 18 μl peptide solution was loaded onto the trap column (100 μmx2.0 mm, Acclaim PepMap C18, Thermo Fisher Scientific, San Jose, CA, USA) at a flow rate of 20 μl/min with solvent A for 3 min and then was separated on a Acclaim PepMap C18 reverse phase column (15 cmx75 μm, Thermo Fisher Scientific, San Jose, CA, USA) with a linear gradient. Starting from 2% B (90% acetonitrile, 0.1% formic acid in deionized water) to 40% B in 110 min. The column was re-equilibrated at initial conditions for 10 min. Column flow rate was maintained at 300 nL/min, and column temperature was maintained at 40 °C. The electrospray voltage of 1.9 kV versus the inlet of mass spectrometer was used.

LTQ Orbitrap XL mass spectrometer was operated in the data-dependent mode to switch automatically between MS and MS/MS acquisition. Survey full-scan MS spectra with one microscan (m/z 400–1800) was acquired in the Orbitrap with a mass resolution of 60,000 at m/z 400, followed by MS/MS of the eight most intensive peptide ions in the LTQ analyzer. The automatic gain control (AGC) was set to 10^6 ions, with injection time of 500 ms. For MS/MS, an isolation window of 3 m/z was used, and the AGC of LTQ was set to 20,000 ions, with maximum injection time of 100 ms. Single charge state was rejected, and dynamic exclusion was used with two microscans in 10 s and 90 s exclusion duration. Precursor ions were activated using 35% normalized collision energy at the default activation q of 0.25 and an activation time of 30 ms. The spectrum were recorded with Xcalibur (version 2.0.7) software.

Thermo Scientific Proteome Discoverer software version 1.4 with the MASCOT v2.3.2 search engine was used for all searches against database. The database was the human UniProtKB/Swiss-Prot database (Release 2014_04_10, with 20,264 entries). To reduce false positive identification results, a decoy database containing the reverse sequences was appended to the database. The searching parameters were set up as follows: full trypsin (KR) cleavage with two maximum missed cleavage sites was considered; carbamidomethyl of cysteine was specified as fixed modification; oxidation of methionine was specified as variable modification. The peptide mass tolerance was 10 ppm and the fragment ion tolerance was 0.8 Da. Percolator algorithm was used to control peptide level false discovery rates (FDR) lower than 1%.

3. Result and discussion

3.1. Characterization of NH_2-functionalized macroporous silica

NH_2-MOSF was prepared by modifying the surfaces of MOSF with amino groups. SEM was utilized to investigate the morphologies and structures of MOSF and NH_2-MOSF (Fig. 1 and Supporting Information Fig. SI-1). From the SEM image of NH_2-MOSF, macroporous silica foams with around 100 nm pore diameter were observed. Compared with the SEM image of MOSF, no large aggregate was observed for NH_2-MOSF, suggesting its well-dispersed state. Fourier transform infrared (FTIR) spectroscopy was carried out to characterize the surface modification, as shown in Fig. 1(b). The dominant multi-peaks at 1000–1100 cm^{-1} are typically assigned to Si–O bond and Si–OH groups in the bare

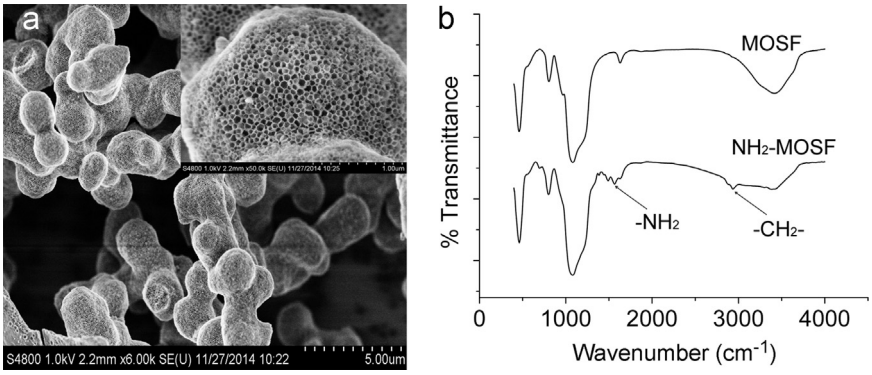

Fig. 1. (a) SEM images (the inset are partially zoomed images of the material) of NH_2-MOSF and (b) FTIR spectra of MOSF and NH_2-MOSF.

MOSF and NH_2-MOSF materials. In the case of NH_2-MOSF, an apparent additional single peak rises around 1580 cm^{-1}, which can be attributed to the grafted $–NH_2$ groups, indicating the successful modification of MOSF with amino groups. The double peaks around 2920 cm^{-1} are due to the existence of $–CH_2–$ groups of silica-coupling agent (APTS). Zeta potential of MOSF and NH_2-MOSF was also examined (data not shown). The unmodified MOSF had the zeta potential of -39 mV because of the abundant silanol groups on the surface of MOSF. In contrast, a new peak appeared at $+18$ mV while the former peak was absent for NH_2-MOSF, indicating the modification of Si–OH by amino groups.

3.2. Tryptic digestion assisted by NH_2-MOSF

Because of its very large pore size and surface modification by amino groups, NH_2-MOSF is used for efficient adsorption of trypsin. The maximum adsorption amount can be achieved within 5 min and the immobilization capacity of trypsin is 100 ng (mg NH_2-MOSF)$^{-1}$, Fig. SI-2 (see Supporting Information).

In a previous study, we have demonstrated that NH_2-MOSF can assist tryptic protein digestion under a wide bulk buffer pH conditions, from pH 3 to 8 [21]. During the proteolysis, proteins and enzyme were quickly adsorbed into the nanopores of NH_2-MOSF, where the amino groups could buffer the local enviroment to an optimum pH to maintain the activity of trypsin for efficient proteolysis independent of bulk buffer pH. Considering the fact that the pH of OGE wells can range from 3 to 10, here we also tested the proteolysis efficiency when using trypsin preloaded NH_2-MOSF in alkaline solution.

At pH 8, the optimum pH for tryptic digestion, the peptide mass fingerprinting (PMF) result of digestion products from 10 min proteolysis of myoglobin assisted by trypsin preloaded NH_2-MOSF is much better than that obtained from 10 min in-solution digestion without NH_2-MOSF, Fig. 2(a and b). 16 finely resolved peptide peaks were observed from the product generated by the NH_2-MOSF assisted digestion, with the sequence coverage of 94% and the MOWSE score of 141. In contrast, the in-solution digestion yielded only 9 peptides identified by MS at poor S/N (sequence coverage: 67%, MOWSE score: 103). These results demonstrate that the reaction rate is significantly accelerated when proteolysis takes place within the nanopores. Under a very alkaline condition at pH 10, the digestion mediated by NH_2-MOSF could still be conducted in 10 min as shown in Fig. 2(d), where 14 high-resolved peptides ($S/N > 10$) were observed, with the sequence coverage of 90%. On the contrary, the in-solution digestion at such a pH condition produced only 8 peptides identified by MS, with the sequence coverage of 64%, Fig. 2(c).

Together with the previous published results [21], it is demonstrated that the trypsin preloaded NH_2-MOSF mediated digestion is very efficient in acidic, neutral, and alkaline media. The amino groups on the surface of NH_2-MOSF make the in-pore local condition appropriate to maintain tryptic activity under any bulk

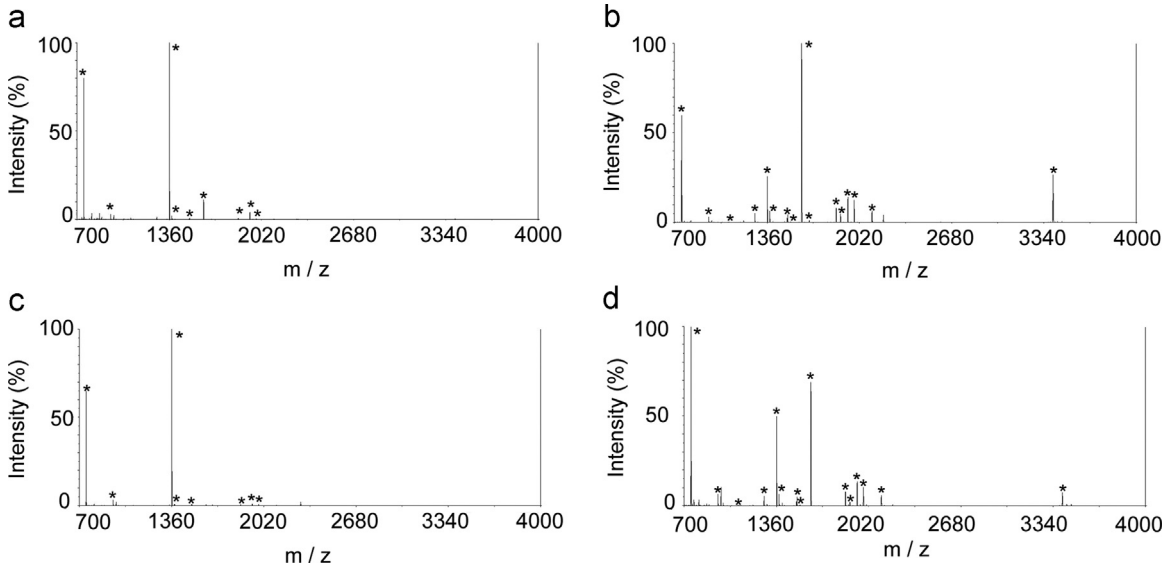

Fig. 2. Mass spectra of the digested products after 10 min of proteolysis (b, d) with or (a, c) without the assistance of NH_2-MOSF in buffers under pH (a, b) 8, and (c, d) 10. *: identified peptides from myoglobin by the peptide mass fingerprinting method.

buffer conditions. The ultrafast reaction rate is attributed to the nano-confinement effect and the *in-situ* enrichment of proteins and enzymes from bulk solutions into the nanopores of NH_2-MOSF [16,23].

3.3. Digestion of proteins during OGE

OGE is a separation technique based on IEF, in which separated compounds are directly recovered in solution. In standard approaches, to obtain a comprehensive analysis of protein-mixture, pre-fractionated proteins by OGE is followed by in-solution proteolysis, and then a second OGE separation of the produced peptides. However, since immobilized pH gradient strips (IPGs) are employed in OGE to generate pH gradients, the protein fractions extracted from different wells have various pH values, which require lyophilisation of fractions and re-dissolving them in ammonium bicarbonate buffer solution (pH~8) before tryptic digestion.

Herein, on-line proteolysis assisted by the trypsin preloaded NH_2-MOSF is carried out during OGE fractionation to save experimental efforts and also to achieve good analysis results, as illustrated in Fig. 3. Under the applied voltage for OGE, proteins are separated and enriched in different wells in accordance with their p*I*, and are digested at the same time by the trypsin preloaded NH_2-MOSF. The digested peptides are then further fractionated by OGE as soon as they are produced. The pre-loading of trypsin in NH_2-MOSF is carried out to ensure that trypsin itself does not migrate during the separation. At the end of the OGE run, focused peptides are directly recovered in solution and collected for subsequent MS analysis or LC–MS analysis.

A standard protein-mixture sample containing myoglobin, beta-casein, cytochrome C, and BSA was used as a model substrate to demonstrate the concept of this protocol. The digested peptides in each OGE well were analysed separately by MALDI–MS, and the identification result was listed in Table SI-1 to SI-4 (see Supporting Information). As can be seen from the tables, 14, 11, 6 and 22 peptides were successfully identified from cytochrome C, myoglobin, beta-casein and BSA, respectively, after OGE separation with on-line NH_2-MOSF assisted proteolysis. The results indicate that the protocol can be used for effective on-line protein digestion and separation.

Based on the proof-of-concept results from protein mixture, we have further applied the proposed system for the large-scale characterization of a biological sample, protein extracts from Hela cell line. The same sample was also studied by the typical OGE method, where the proteins were overnight digested in solution, and then the peptides were collected for OGE fractionation. All the fractions from OGE in both cases were analysed by LC–ESI–MS/MS with the same protocol.

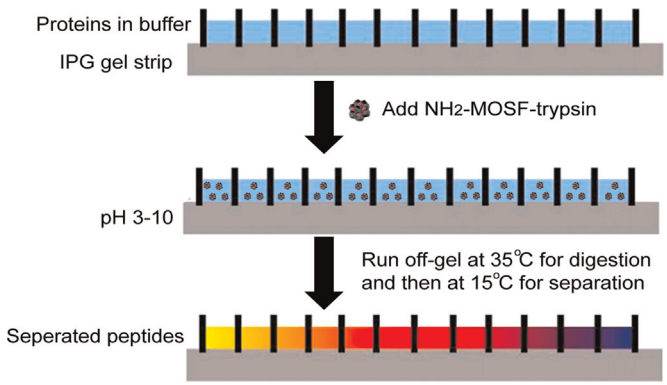

Fig. 3. Schematic illustration of on-line proteolysis assisted by trypsin preloaded NH_2-MOSF during OGE separation.

Fig. 4(a) compares the peptides identified from fraction no.8, no.18, and the summed results from all fractions by both strategies. There were 2903 peptides detected from the no.8 fraction by the NH_2-MOSF assisted on-line digestion during OGE system, while only 667 peptides were obtained from the same fraction when in-solution digestion followed with OGE was applied. There were fewer peptides obtained from the fraction with alkaline pH. 1105 peptides were achieved by the NH_2-MOSF assisted on-line digestion from the faction no.18, and even a smaller amount of peptides (262) were identified by the in-solution digestion method, indicating that most of the peptides digested from Hela cell protein extracts have acidic p*I*. Since each protein can generate many peptides distributed in various wells, we performed the protein identification by combining peptides data from all wells. Fig. 4(b) shows that 1877 proteins (from 5629 peptides) were obtained by the in-solution digestion method. 90% of them were also detected by the NH_2-MOSF assisted on-line digestion method, which resulted in a total of 3592 proteins (from 18,912 peptides) identified.

There is a significant increase in the number of identified proteins by the NH_2-MOSF assisted on-line digestion method. The presence of one protein species at high concentration usually suppresses the digestion of low abundance proteins. Pre-fractionation of biological samples can greatly reduce their complexity, thereby improving the detection results. However, one critical aspect of the multi-separation steps is the risk to lose sample during the fractionation and collection procedures. In the present case, proteins are fractionated and digested directly without extra procedure, which dramatically shortens the time required, and avoids the risk of sample loss, while keeps the advantage of sample simplification before/during proteolysis. On the other hand, during the material assisted proteolysis, proteins are trapped together with trypsin at high local concentration in the nanopores of NH_2-MOSF, which also promotes the digestion of low abundance proteins The significant increase in the number of identified proteins indicates that the NH_2-MOSF assisted on-line proteolysis during OGE separation is a beneficial tool for proteome research.

The details of the proteins identified by both strategies are displayed in Fig. 5 and Table SI 5 and SI 6 (see Supporting Information), which show the distribution of p*I* and MW of all the proteins. The MW of the proteins identified with the NH_2-MOSF strategy is mainly between 10 kDa and 100 kDa (Fig. 5(a and c)), and the *pI* (Fig. 5(a and d)) is ranged from 3.78 to 12.15, and mainly between 5 and 7. The distribution of p*I* and MW of all the proteins achieved from the in-solution digestion are also displayed in Fig. 5 (b–d), which has the same pattern as the results obtained from the NH_2-MOSF assisted digestion. In each fraction of p*I* and MW, the NH_2-MOSF assisted digestion during OGE shows enhanced identification of proteins, indicating that the protocol can be universally applied to protein samples with any p*I* and MW.

4. Conclusion

The functional NH_2-MOSF assisted proteolysis has been successfully applied to the on-line separation and digestion of proteins during OGE. The in-pore local microenvironment buffered by amino group is suitable for tryptic reactions in the presence of acidic or alkaline bulk solutions, which makes it possible to overcome the dilemma of trypsin inactivity in Off-Gel buffer. Using the developed protocol, enhanced digestion and identification efficiency are achieved in a short time. The NH_2-MOSF assisted protocol is extremely simple to use, and circumvents the time consuming aspect of protein digestion before OGE-LC–MS analysis. Furthermore, the successful utilization of such an approach to the

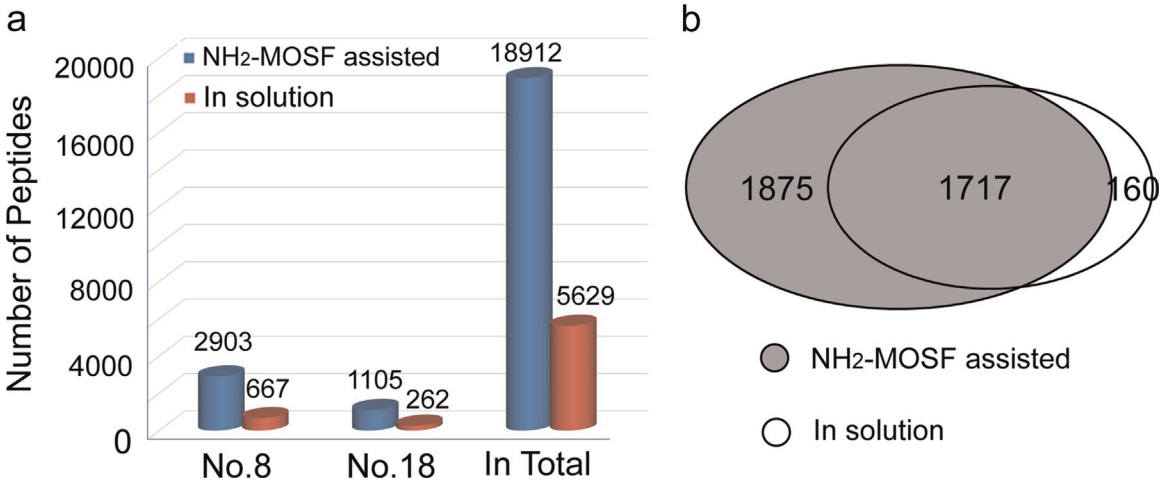

Fig. 4. (a) Comparison of the number of peptides identified with the NH_2-MOSF assisted digestion during OGE strategy (blue) and the standard in-solution digestion followed with OGE strategy (red). (b) Overlap of the overall identified proteins by the two methods. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

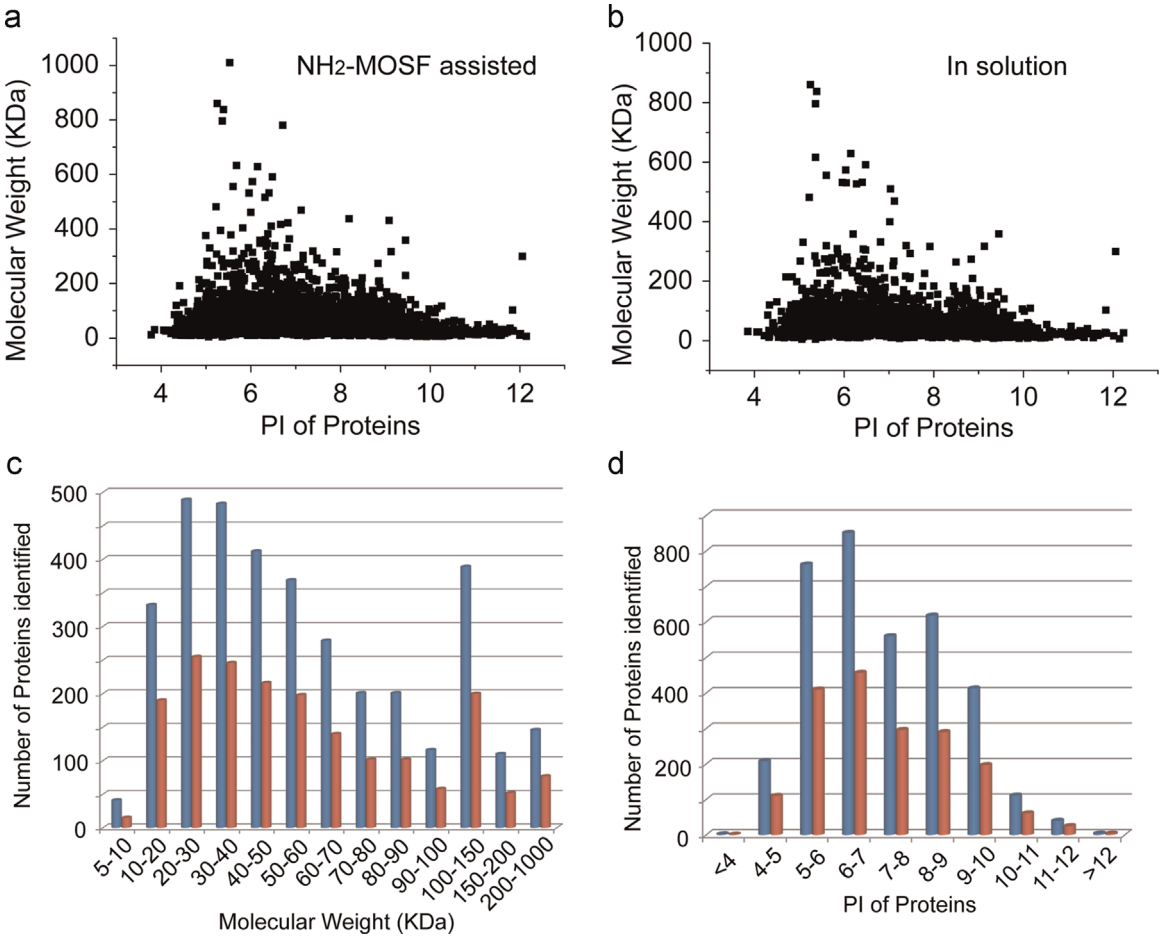

Fig. 5. The distribution of MW and p*I* values of the identified proteins by using (a) NH_2-MOSF assisted on-line digestion method, and (b) traditional in-solution digestion method. Comparison of the (c) MW and (d) p*I* distribution of the proteins identified with the NH_2-MOSF assisted on-line digestion during OGE (blue) and standard in-solution digestion followed with OGE (red). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

complex sample indicates its potential application in large-scale proteome research.

Conflict of interest statement

The authors have declared no conflict of interest.

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Appendix A. Supplementary material

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