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Monolithic Capillary Column Based Glycoproteomic Reactor for High-Sensitive Analysis of N-Glycoproteome

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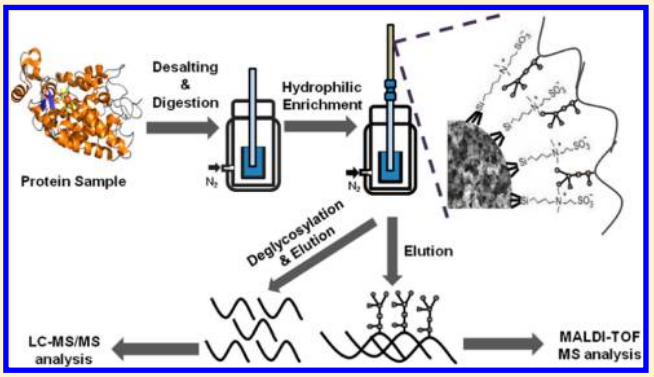
Supporting Information

ABSTRACT: Despite the importance of protein N-glycosylation in a series of biological processes, in-depth characterization of protein glycosylation is still a challenge due to the high complexity of biological samples and the lacking of highly sensitive detection technologies. We developed a monolithic capillary column based glycoproteomic reactor enabling high-sensitive mapping of N-glycosylation sites from minute amounts of sample. Unlike the conventional proteomic reactors with only strong-cation exchange or hydrophilic-interaction chromatography columns, this novel glycoproteomic reactor was composed of an 8 cm long C12 hydrophobic monolithic capillary column for protein digestion and a 6 cm long organic−silica hybrid hydrophilic monolithic capillary column for glycopeptides enrichment and deglycosylation, which could complete whole-sample preparation including protein purification/desalting, tryptic digestion, enrichment, and deglycosylation of glycopeptides within about 3 h. The developed reactor exhibited high detection sensitivity in mapping of N-glycosylation sites by detection limit of horseradish peroxidase as low as 2.5 fmol. This reactor also demonstrated the ability in complex sample analysis, and in total, 486 unique N-glycosylation sites were reliably mapped in three replicate analyses of a protein sample extracted from ~10^4 HeLa cells.



Protein glycosylation is one of the most important post-translational modifications and plays crucial roles in a series of physiological and pathological processes, such as cell−cell recognition and communication, protein folding, stabilization, and translocation.[1−3] N-linked glycans, with the oligosaccharides attached to asparagine (Asn) residues in a consensus sequence of N-X-S/T (where X can be any amino acid except proline) or rarely the N-X-C motif in membrane and extracellular proteins, is the most common type of protein-linked glycan. The attachment of N-linked glycans has a strong influence on protein structure and function. Aberrant N-glycosylation on some important proteins is highly associated with various types of cancers.[4−7] N-Glycosylated proteins are also important for disease diagnosis, prognosis, and therapeutic response to drugs. Consequently, characterization of N-glycosylation sites is one of the key issues not only for biological and biomedical research but also for the pharmaceutical and biotechnology industries.[8,9]

Characterization of protein N-glycosylation in a complex biological sample such as plasma/serum or cell lysates is still a big challenge due to the enormous complexity of protein samples, vast dynamic range of protein concentrations, and relative low abundance and low ionization efficiency of N-glycosylated peptides.[10,11] Various methods has been developed for in-depth characterization of the N-glycoproteome, such as hydrazide chemistry for human plasma and urine,[12,13] filter-aided sample preparation for mouse plasma and other organs,[9] and so forth. However, usually a large amount of staring material is required for these methods, which limits their application for analysis of minute amounts of samples, such as clinical samples. Recently, the proteomic reactor emerges as a promising technique for proteome analysis with high sensitivity and easiness to automation. A few types of reactors specialized for protein N-glycosylation analyses have been developed. Zhou et al. integrated ^{18}O labeling, deglycosylation, digestion, and peptide fractionation of the enriched N-glycosylated proteins on an 8 cm long strong cation-exchange (SCX) column for glycoproteome analysis.[14] However, this SCX column based reactor has no capability for N-glycosylated proteins or peptide enrichment. We developed a centrifugation-assisted microreactor that integrated trypsin digestion, hydrophilic-interaction chromatography (HILIC) enrichment, and on-column deglycosylation for N-glycoproteome analysis,[15] and 92 N-glycosylation sites were identified from only 10 nL of human serum. But, desalting using a centrifugal membrane filter may also lose proteins with lower molecular weights, and the

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analysis throughput and detection sensitivity still needs to be improved to meet the demand of in-depth N-glycoproteome analysis.

In this study, we developed a monolithic column based glycoproteomic reactor, which allows facile integration of protein purification/desalting, tryptic digestion, enrichment, and deglycosylation of N-glycosylated peptides, for glycoproteome analysis of minute amounts of samples. This glycoproteomic reactor was composed of an 8 cm long C12 hydrophobic monolithic capillary column for protein digestion and a 6 cm long hybrid HILIC monolithic capillary column for N-glycosylated peptide enrichment and deglycosylation. The detection limit of horseradish peroxidase (HRP) as low as 2.5 fmol was achieved with this N-glycoproteomic reactor, and the processing time could be shortened to about 3 h. Finally, 486 unique N-glycosylation sites were reliably mapped by three replicate analyses of 1 μg of protein sample extracted from $\sim 10^4$ HeLa cells.

EXPERIMENTAL SECTION

Reagents and Materials. Immunoglobulin G from human (IgG), horseradish peroxidase (HRP), trypsin, iodoacetamide (IAA), dithiothreitol (DTT), trifluoroacetic acid (TFA), ammonium bicarbonate (NH_4HCO_3), [2-(methacryloyloxy)-ethyl]dimethyl-(3-sulfopropyl) ammonium hydroxide (MSA), γ-methacryloxypropyltrimethoxysilane (γ-MAPS), poly-(ethylene glycol) (PEG, M_n = 10 000), lauryl methacrylate (LMA), and ethylene dimethacrylate (EDMA) were purchased from Sigma (St. Louis, MO). Tetramethoxysilane (TMOS) was purchased from Chemical Factory of Wuhan University (Wuhan, China). Ethylene diamine tetraacetic acid (EDTA), ethylene glycol bis(2-aminoethyl) tetraacetic acid (EGTA), and phenylmethanesulfonyl fluoride (PMSF) were purchased from Amresco (Solon, OH). Azobisisobutyronitrile (AIBN) was purchased from Shanghai Chemical Plant (Shanghai, China) and recrystallized in ethanol before use. PNGase F was purchased from New England Biolabs (Ipswich, MA), and formic acid (FA) was from Fluka (Buches, Germany). High-performance liquid chromatography (HPLC)-grade acetonitrile (ACN) was from Merck (Darmstadt, Germany). Fused silica capillaries with dimensions of 75 and 200 μm i.d. were obtained from Yongnian Optical Fiber Factory (Hebei, China). C18 AQ beads (3 and 5 μm, 120 Å) were purchased from Daiso (Osaka, Japan). All the water used in experiments was purified with a Milli-Q system from Millipore (Milford, MA). Other chemical reagents were all of analytical grade.

Preparation of C12 Monolithic Column. The capillary was pretreated with γ-MAPS as in our previous report.[16] Then, lauryl methacrylate (100 μL), ethylene dimethacrylate (100 μL), 1-propanol (170 μL), 1,4-butanediol (130 μL), and AIBN (2 mg) was mixed and sonicated for 5 min to obtain a homogeneous solution and then filled into the pretreated capillary, followed with incubation in a water bath at 60 °C for 12 h by keeping the column sealed at both ends with rubber plugs. Finally, the monolithic capillary column was washed with methanol using a HPLC pump to remove unreacted monomers and other residuals.

Preparation of HILIC Monolithic Column. The hydrophilic monolith was prepared according to the "one-pot" process reported previously.[17] Briefly, acetic acid (0.01 M, 5.0 mL), PEG (M_n = 10 000, 540 mg), urea (800 mg), TMOS (1.8 mL), and γ-MAPS (500 μL) were mixed together and stirred for 2.5 h at 0 °C to form a homogeneous solution. Then, 40 mg of MSA and 2 mg of AIBN were added into 0.5 mL of the hydrolyzed prepolymerization mixture, followed with a 10 min sonication for mixing and degassing. Then, this mixture was manually injected into the pretreated capillary to an appropriate length by a syringe. After sealing of both ends, the capillary was incubated at 40 °C for 12 h for simultaneous condensation and polymerization. The obtained hybrid monolithic capillary column was then sequentially flushed with water and methanol to remove the unreacted residuals.

Sample Preparation. The HeLa cells were cultured in RPMI-1640 medium, supplemented with 10% bovine serum, 100 U/mL penicillin and streptomycin in a humidified atmosphere of 5% CO_2 at 37 °C. The cells were harvested at about 80% density, and the cell pellets were softly homogenized in an ice-cold lysis buffer containing 8 M urea, 50 mM Tris–HCl (pH = 7.4), 65 mM DTT, 1% protease cocktail (v/v), 1% Triton X-100 (v/v), 1 mM EDTA, 1 mM EDGA, and 1 mM PMSF, sonicated for 400 W × 120 s, and centrifuged at 25 000g for 1 h. Then, the supernatant was precipitated with 5 volumes of ice-cold acetone/ethanol/acetic acid (v/v/v = 50/50/0.1) at −20 °C overnight. The protein precipitant was centrifuged at 15 000g for 30 min, and the pellet was washed separately with acetone and 75% ethanol. After being lyophilized to dryness, the HeLa cell proteins were redissolved into buffer containing 100 mM NH_4HCO_3 (pH = 8.2) and 8 M urea. The protein concentration was determined by Bradford assay, and the mixture was reduced by 10 mM DTT at 60 °C for 1 h and then alkylated by 20 mM IAA in the dark at room temperature for 30 min. After that, the protein concentration was diluted to 1 μg/μL with 100 mM NH_4HCO_3 buffer (pH = 8.2) and stored at −20 °C.

For standard sample preparation, 100 μg of HRP protein was denatured by 100 μL of 8 M urea/100 mM NH_4HCO_3 and stored at −20 °C.

A 1 mg amount of IgG digest was obtained as described previously.[15] Lyophilized IgG digest was redissolved in loading buffer.

Optimization of HILIC Enrichment Conditions for Glycopeptides. First, 1 μg of IgG tryptic digest (20 μL) dissolved in different loading buffers (0.5% TFA/80% ACN, 0.2% TFA/80% ACN, 0.1% TFA/80% ACN, or 0.1% FA/80% ACN) was loaded onto the HILIC monolithic column. Then, the HILIC column was washed with 20 μL of loading buffer. Finally, the glycopeptides were eluted with 20 μL of 0.1% FA and analyzed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS).

Monolithic Capillary Column Based Glycoproteomic Reactor. The monolithic capillary column based glycoproteomic reactor was achieved by integrating a 200 μm i.d. × 8 cm long C12 monolithic column (enzyme reactor) and a 200 μm i.d. × 6 cm long HILIC monolithic column (glycopeptide enrichment reactor) with a zero dead volume (ZDV) union.

HRP protein (1 μL) or HeLa cell proteins solution (1 μg/μL) were acidified by 18 μL of 0.2% FA, mixed with trypsin at a mass ratio of 2.5:1, and loaded onto the C12 monolithic column by nitrogen pressure. After being washed with 20 μL of 0.1% FA for desalting and 1.5 μL of 100 mM NH_4HCO_3 separately, the capillary column was dried by nitrogen gas. Enzyme digestion was initiated through filling the monolithic column with 80% ACN/6 mM NH_4HCO_3. After 2 h of digestion in a water bath at 37 °C by keeping the column sealed with rubber plugs, the C12 monolithic column was joined with the hydrophilic column, and the peptides were eluted to the

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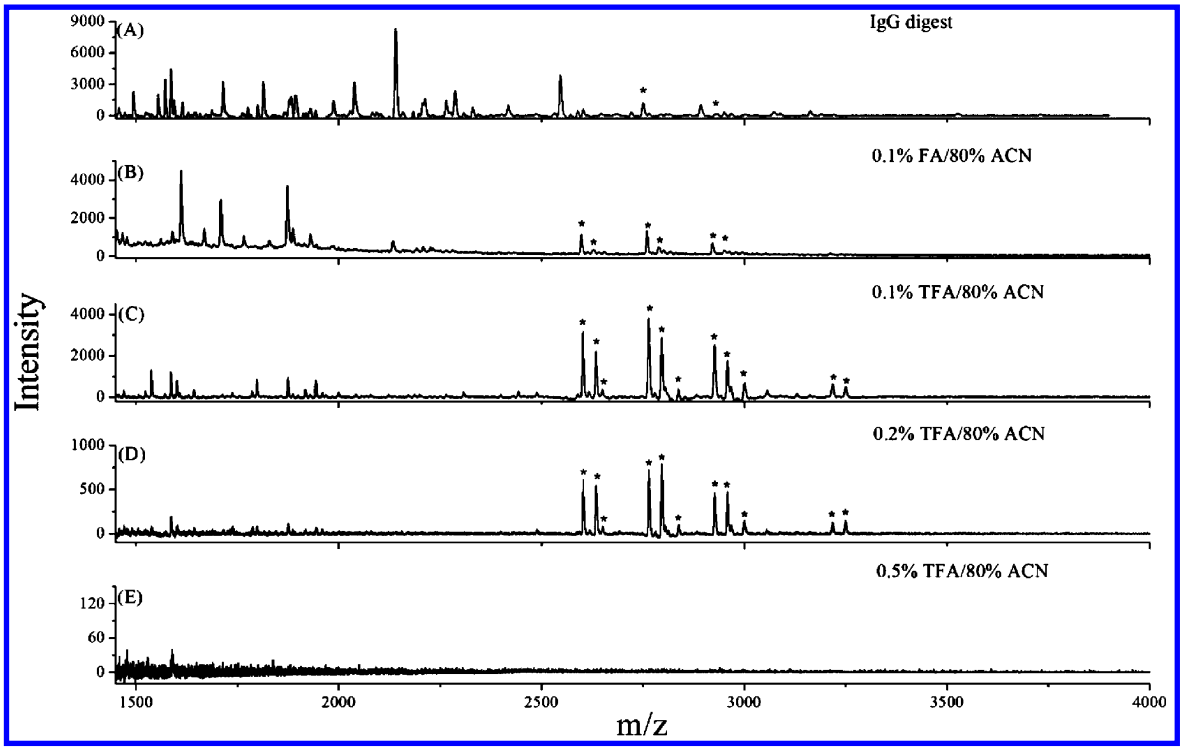

Figure 1. MALDI-TOF mass spectra of 25 ng of IgG digest (A) before enrichment and (B–E) after enrichment by a HILIC monolithic column with different loading buffers. The glycopeptides were marked with an "*".

HILIC monolithic column by using 40 μL (for HRP samples) and 100 μL (for HeLa cell samples) of 0.2% TFA/80% ACN, respectively. Finally, the enriched glycopeptides were eluted with 20 μL of 0.1% FA for MALDI-TOF MS analysis directly (for HRP samples) or 20 μL of 20 mM NH_4HCO_3 containing 100 U PNGase F to deglycosylation of the enriched glycopeptides for LC-MS/MS analysis (for HeLa cell samples).

Conventional in-solution digestion was also performed for comparison. A 1 μg amount of denatured HRP protein was diluted with 100 mM NH_4HCO_3 and digested at 37 °C for 16 h with a protein to trypsin ratio of 10:1. The digest was desalted using Millipore ZipTips and eluted with 20 μL of 0.2% TFA/80% ACN. The glycopeptides were enriched by the HILIC monolithic column and subsequently analyzed by MALDI-TOF MS.

MS Analysis. Standard protein samples were analyzed by an AB Sciex 5800 MALDI-TOF/TOF mass spectrometer (AB Sciex, CA) equipped with a pulsed Nd/YAG laser at 355 nm in linear positive ion mode. A 0.5 μL amount of the eluate and 0.5 μL of matrix (25 mg/mL 2,5-dihydroxy-benzoic acid in 50% ACN/H_2O) were spotted on the MALDI plate for MS analysis.

HeLa cell samples were analyzed by a LTQ Orbitrap Velos (Thermo, San Jose, CA) with an Accela 600 HPLC system (Thermo, San Jose, CA) for separation. The deglycosylated peptides were loaded on a C18 capillary trap column (200 μm i.d. × 4 cm) packed with C18 AQ beads (5 μm, 120 Å). The separation of deglycopeptides was performed on an analytical column (75 μm i.d. × 15 cm) packed with C18 AQ beads (3 μm, 120 Å). The buffers used for online analysis were 0.1% (v/v) formic acid in water and 0.1% (v/v) formic acid in ACN, and the flow rate was 200 nL/min for HPLC-MS/MS during analysis. The gradient from 5% to 40% (v/v) ACN was performed in 95 min. The MS and MS/MS spectra were collected by collision-induced dissociation at 35% energy in a data-dependent mode with one MS scan followed by 20 MS/MS scans. The resolution was set at 60 000 for full MS, and the scan range was set from m/z = 400 to 2000.

Database Searching. Data files were searched with MaxQuant (1.1.1.36) against the IPI human database (v3.80). Cysteine carboxamidomethylation was set as a static modification of 57.0215 Da; methionine oxidation and asparagine deamination were set as variable modifications of 15.9949 and 0.9840 Da, respectively. Mass tolerances were 20 ppm and 0.5 Da for the parent and fragment ions, respectively. A maximum of two missed cleavages was allowed, and the false positive rates (FDRs) were controlled to <1% for identification of peptides, proteins, and glycosylation sites.

RESULTS AND DISCUSSION

Enrichment of N-Glycosylated Peptides by Hydrophilic Monolithic Column. The enrichment performance of N-glycosylated peptides by using a hydrophilic monolithic column was evaluated at first. The selectivity of nonglycosylated peptides and glycosylated peptides on the hydrophilic solid phase could be significantly improved by adding ion-pairing reagents, such as TFA.[11,15] Therefore, different types of sample loading buffers with or without TFA were applied for enrichment of N-glycosylated peptides of an IgG digest by a 200 μm i.d. × 6 cm hydrophilic monolithic column followed by MALDI-TOF MS analysis. A few nonglycopeptides from IgG could still be detected with considerable intensity if 0.1% FA/80% ACN or 0.1% TFA/80% ACN was utilized as sample loading buffers (Figure 1B,C). However, only N-glycosylated peptides could be observed when the TFA concentration was increased to 0.2% (Figure 1D). However, almost no peptide was detected when the TFA concentration was increased to 0.5% (Figure 1E). This might be attributed to the fact that the existence of a high concentration of TFA could gradually

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destroy the silica-based structure of the monolithic column. Therefore, 0.2% TFA/80% ACN was chosen as the sample loading buffer in the following experiments to ensure the enrichment selectivity of N-glycosylated peptides.

Evaluation of the Performance of the Glycoproteomic Reactor. In enzyme-adsorption type enzyme reactors, strong cation- or anion-exchange (SAX) materials are used to adsorb the proteins and enzyme at low or high pH at first, and the proteins are digested by adjusting the pH to activate the enzyme.[14,18] However, high salt aqueous solutions are usually used to elute the digested peptides from the SCX or SAX columns, which is incompatible with the following hydrophilic column enrichment of N-glycosylated peptides by HILIC materials. It is known that the protein digestion could be accelerated in a high percentage of ACN solution,[19] which is also a nice loading buffer for HILIC enrichment of N-glycosylated peptides. Therefore, a C12 RP monolithic column was used as a reactor for fast protein digestion in buffer with a high concentration of ACN and then eluted to the HILIC monolithic column for enrichment of N-glycosylated peptides in this study. Briefly, the sample was acidified, diluted by formic acid solution, mixed with trypsin, and loaded onto the C12 section of the monolithic column at first. After desalting by 0.1% FA solution and alkalifying the column by ammonium bicarbonate solution, protein digestion was initiated through filling the microreactor with 80% ACN/6 mM NH_4HCO_3. Then, the C12 monolithic column was joined with the hydrophilic monolithic column, and the N-glycopeptides were captured selectively when the digests were eluted to the HILIC monolithic column by 80% ACN/0.2% TFA. Finally, the captured N-glycopeptides were eluted with 0.1% FA aqueous solution or NH_4HCO_3 buffer solution containing PNGase F for deglycosylation.[15] Therefore, we developed a RP−HILIC monolithic columns based glycoproteomic reactor, which fully integrated the sample preparation procedures of desalting, tryptic digestion, enrichment, and deglycosylation of N-glycosylated peptides together as described above (Figure 2).

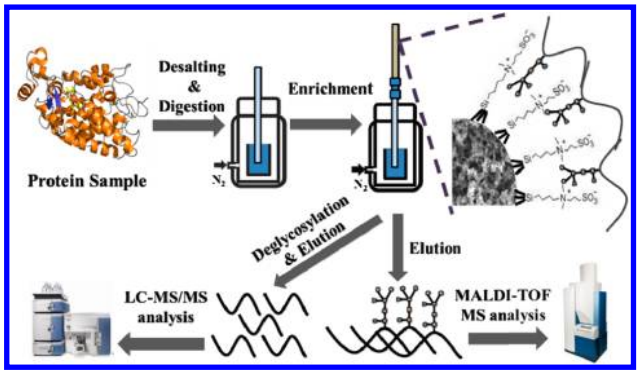

Figure 2. Seamless workflow for glycoproteome analysis with the novel monolithic glycoproteomic reactor.

The performance of this glycoproteomic reactor was evaluated by using HRP as the standard N-glycosylated protein because it has a number of N-glycosylation sites (Supporting Information).[20] For comparison, an in-solution digestion approach was also applied. All of the nine glycosylation sites could be detected by both the glycoproteomic reactor based and in-solution digestion based strategies when the amount of HRP is higher than 25 fmol (Figure 3). However, four of the nine N-glycosalyation sites could be still detected by the glycoproteomic reactor based approach when only 2.5 fmol of HRP was utilized, in contrast to only one N-glycosylation site being detected by the in-solution digestion strategy (Figure 3E,J). Comparable peak intensities and ratios of signal-to-noise (S/N) could be observed by both strategies when more than 25 fmol of HRP was applied. However, the peak intensity and S/N were much higher with the glycoproteomic reactor based strategy than those with the in-solution digestion strategy when less than 25 fmol of HRP was used (Figure 3C−E and H−J). Obviously, this RP−HILIC monolithic column based glycoproteomic reactor exhibits much higher detection sensitivity and is more suitable for N-glycoproteome analysis of minute amounts of protein samples.

N-Glycoproteome Analysis of HeLa Cell Proteins. The RP−HILIC monolithic column based glycoproteomic reactor was further applied to high sensitive N-glycoproteome analysis of a complex HeLa cell lysate. A 1 μg amount of protein sample extracted from ~10^4 HeLa cells was analyzed as described above. After controlling the FDR <1% for identification of N-glycosylated peptides, 289, 315, and 370 unique N-glycosylation sites could be feasibly identified in three independent LC-MS/MS analyses using 95 min binary gradient elution, respectively. The numbers of corresponding N-glycosylated proteins identified were 178, 189, and 214, respectively. 486 unique N-glycosylation sites from 279 N-glycosylated proteins were identified in total, among which 173 unique N-glycosylation sites were identified in all three runs and 315 unique N-glycosylation sites were identified in at least two runs (Figure 4). Among the 486 unique N-glycosylation sites, 377 unique N-glycosylation sites from 215 N-glycosylated proteins are in a consensus sequence of N-X-S/T (where X can be any amino acid except proline) or rarely the N-X-C motif. Thus, these 377 N-glycosylation sites can be considered as highly reliable sites. 294 unique N-glycosylation sites among them have been annotated as known sites that have been reported previously, and 83 (22.0%) N-glycosylation sites are novel. Therefore, the above results demonstrated the high sensitivity of the RP−HILIC monolithic column based glycoproteomic reactor for N-glycoproteome analysis of minute amounts of samples.

In conventional in-solution digestion approaches for glycoproteome analysis, protein samples are first digested in buffer solution containing a high concentration of urea, followed with desalting and lyophilization, and finally, a small part of the purified digest is applied for enrichment of N-glycosylated peptides and nanoflow LC-MS/MS analysis. The tedious procedures of the in-solution digestion approach may cause significant sample loss, which will be more conspicuous when a minute amount of starting material is analyzed. In our glycoproteomic reactor based approach, most of the sample preparation procedures are integrated into a glycoproteomic reactor, and the whole process is faster and more efficient. The processing time is as short as ~3 h, which is much faster than the conventional in-solution digestion approach that may consume ~2 days. Therefore, both high throughput and high sensitivity could be feasibly obtained for N-glycoproteome analysis by using this RP−HILIC monolithic capillary column based glycoproteomic reactor.

■ CONCLUSIONS

In summary, a novel RP−HILIC monolithic capillary column based glycoproteomic reactor, which integrates multiple procedures of the sample preparation, has been developed.

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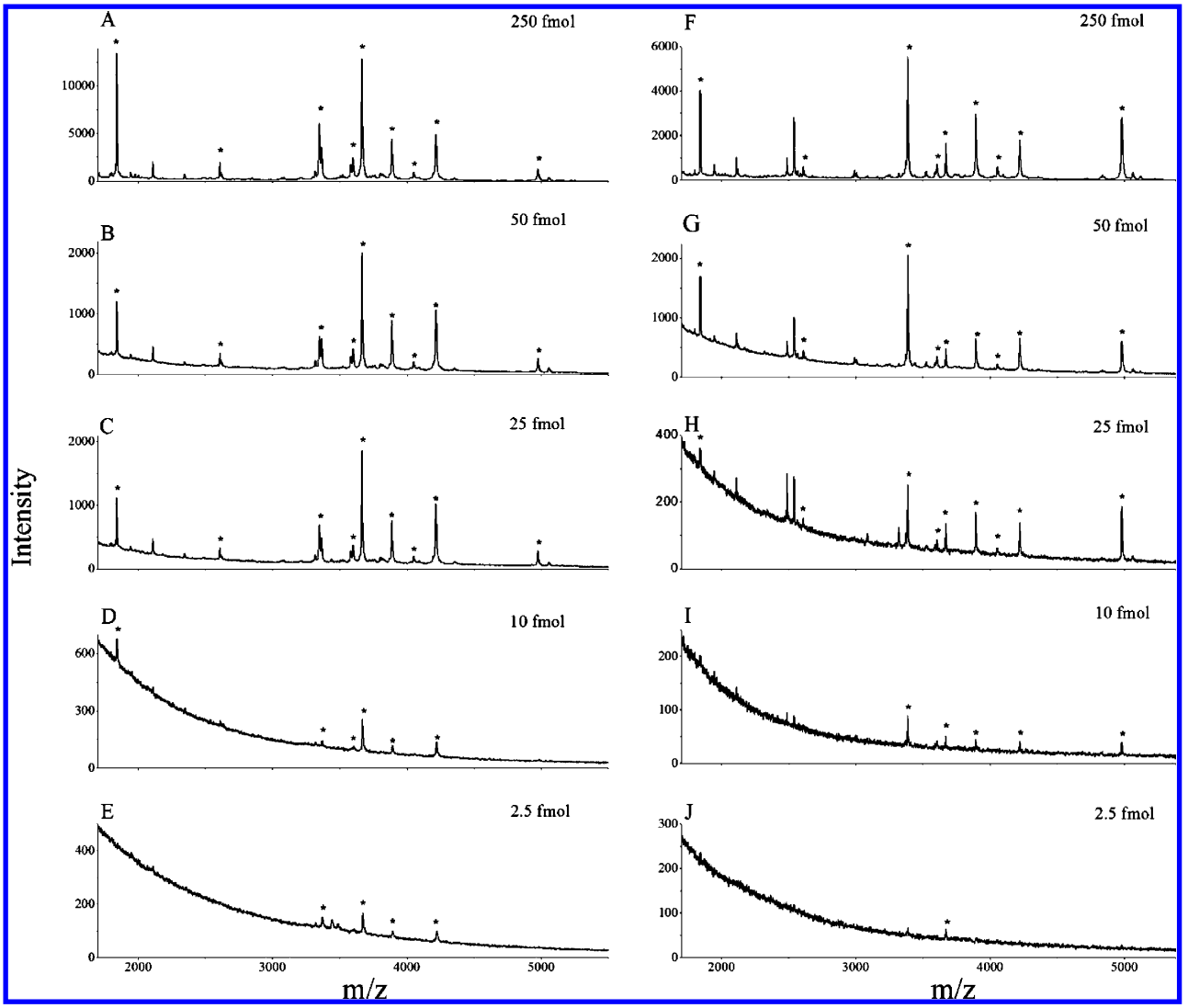

Figure 3. Evaluation of detection sensitivity for analysis of HRP by using the method with (A–E) the monolithic capillary column based glycoproteomic reactor and (F–J) the in-solution digestion method. Different amounts of HRP were used, and the eluted glycopeptides (marked with an "*") were detected by MALDI-TOF MS directly.

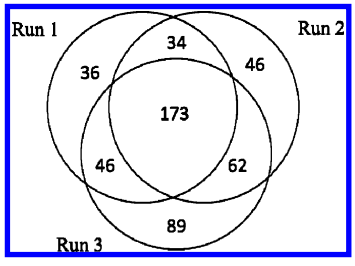

Figure 4. Overlap of the glycosylation sites identified by glycoproteome analysis of 1 μg of HeLa cell proteins from three one-dimensional LC-MS/MS analyses.

We applied a C12 hydrophobic monolithic column as a reactor for fast protein sample digestion and a hydrophilic monolithic column for enrichment of N-glycosylated peptides. This glycoproteomic reactor exhibited much higher detection sensitivity and analysis throughput than conventional in-solution digestion approaches for N-glycoproteome analysis. Finally, 486 unique N-glycosylation sites in 279 N-glycosylated proteins were feasibly identified from three parallel LC-MS/MS analyses of 1 μg of protein sample extracted from ~10^4 HeLa cells. This novel RP–HILIC monolithic capillary column based glycoproteomic reactor exhibited great potential for high-sensitivity N-glycoproteme analysis of minute amounts of protein samples.

■ ASSOCIATED CONTENT

Supporting Information

Glycosylation sites of HPR and the corresponding mass of the glycopeptides; additional information for glycoproteome analysis of HeLa cell proteins; and abbreviations and acronyms used within the text. 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.

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■ REFERENCES

(1) Helenius, A.; Aebi, M. *Science* **2001**, *291* (5512), 2364−2369.
(2) Lowe, J. B. *Cell* **2001**, *104* (6), 809−812.
(3) Ohtsubo, K.; Marth, J. D. *Cell* **2006**, *126* (5), 855−867.
(4) Brooks, S. A.; Carter, T. M.; Royle, L.; Harvey, D. J.; Fry, S. A.; Kinch, C.; Dwek, R. A.; Rudd, P. M. *Anti-Cancer Agents Med. Chem.* **2008**, *8* (1), 2−21.
(5) Kobata, A. *Pigm. Cell Res.* **1989**, *2* (4), 304−308.
(6) Kobata, A.; Amano, J. *Immunol. Cell Biol.* **2005**, *83* (4), 429−439.
(7) Woods, R. J.; Edge, C. J.; Dwek, R. A. *Nat. Struct. Biol.* **1994**, *1* (8), 499−501.
(8) Lazar, I. M.; Lazar, A. C.; Cortes, D. F.; Kabulski, J. L. *Electrophoresis* **2011**, *32* (1), 3−13.
(9) Zielinska, D. F.; Gnad, F.; Wisniewski, J. R.; Mann, M. *Cell* **2010**, *141* (5), 897−907.
(10) Pan, S.; Chen, R.; Aebersold, R.; Brentnall, T. A. *Mol. Cell. Proteomics* **2011**, *10* (1), mcp.R110.003251-1−mcp.R110.003251-14.
(11) Mysling, S.; Palmisano, G.; Hojrup, P.; Thaysen-Andersen, M. *Anal. Chem.* **2010**, *82* (13), 5598−5609.
(12) Ishihara, T.; Fukuda, I.; Morita, A.; Takinami, Y.; Okamoto, H.; Nishimura, S. I.; Numata, Y. *J. Proteomics* **2011**, *74* (10), 2159−2168.
(13) Halim, A.; Nilsson, J.; Ruetschi, U.; Hesse, C.; Larson, G. *Mol. Cell. Proteomics* **2012**, *11* (4), M111.013649.
(14) Zhou, H.; Hou, W. M.; Denis, N. J.; Zhou, H. J.; Vasilescu, J.; Zou, H. F.; Figeys, D. *J. Proteome Res.* **2009**, *8* (2), 556−566.
(15) Zhu, J.; Wang, F. J.; Chen, R.; Cheng, K.; Xu, B.; Guo, Z. M.; Liang, X. M.; Ye, M. L.; Zou, H. F. *Anal. Chem.* **2012**, *84* (11), 5146−5153.
(16) Wu, R. A.; Zou, H. F.; Ye, M. L.; Lei, Z. D.; Ni, J. Y. *Anal. Chem.* **2001**, *73* (20), 4918−4923.
(17) Lin, H.; Ou, J. J.; Zhang, Z. B.; Dong, J.; Wu, M. H.; Zou, H. F. *Anal. Chem.* **2012**, *84* (6), 2721−2728.
(18) Zhou, H. J.; Elisma, F.; Denis, N. J.; Wright, T. G.; Tian, R. J.; Zhou, H.; Hou, W. M.; Zou, H. F.; Figeys, D. *J. Proteome Res.* **2010**, *9* (3), 1279−1288.
(19) Russell, W. K.; Park, Z. Y.; Russell, D. H. *Anal. Chem.* **2001**, *73* (11), 2682−2685.
(20) Wuhrer, M.; Hokke, C. H.; Deelder, A. M. *Rapid Commun. Mass Spectrom.* **2004**, *18* (15), 1741−1748.