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Integrated Sample Pretreatment System for N-Linked Glycosylation Site Profiling with Combination of Hydrophilic Interaction Chromatography and PNGase F Immobilized Enzymatic Reactor via a Strong Cation Exchange Precolumn

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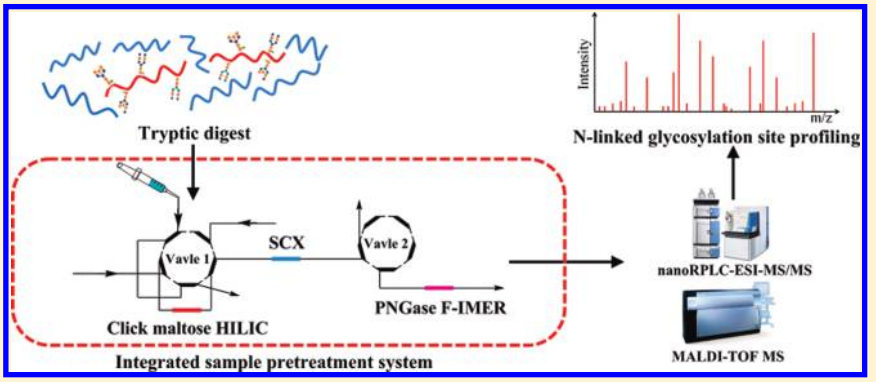
Supporting Information

ABSTRACT:



An integrated sample pretreatment system, composed of a click maltose hydrophilic interaction chromatography (HILIC) column, a strong cation exchange (SCX) precolumn, and a PNGase F immobilized enzymatic reactor (IMER), was established for the simultaneous glycopeptide enrichment, sample buffer exchange, and online deglycosylation, by which the sample pretreatment for glycoproteome could be performed online automatically, beneficial to improve the efficiency and sensitivity of the N-linked glycosylation site identification. With such a system, the deglycosylated glycopeptide from the digests of avidin with the coexistence of 50 times (mass ratio) BSA could be selectively detected, and the detection limit as low as 5 fmol was achieved. Moreover, the sample pretreatment time was significantly shortened to ~1 h. Such a system was further successfully applied for analyzing the digest of the soluble fraction extracted from rat brain. A total of 120 unique glycoprotein groups and 196 N-linked glycosylation sites were identified by nanoreversed phase liquid chromatography−electrospray ionization-tandem mass spectrometry (nanoRPLC-ESI-MS/MS), with the injected digests amount as 6 μg. All these results demonstrate that the integrated system is of great promise for N-linked glycosylation site profiling and could be further online coupled with nanoHPLC-ESI-MS/MS to achieve high-throughput glycoproteome analysis.

N-glycosylation is one of the most common and complex post-translational modification of proteins and plays an important role in cell−cell interaction, signal transduction, cancer immunology, and so forth.[1−4] The global mapping of the N-linked glycosylation site, which generally falls into the canonical N-!P-S/T (where !P denotes any amino acid except proline) sequence motif, is a prerequisite for fully understanding the biological functions of N-linked glycoproteins. Mass spectrometry (MS) coupled with chromatography has become an effective tool for analyzing N-linked glycoproteins and glycosylation sites in particular.[5−9] However, the low abundance of glycopeptides relative to the large excess of nonglycosylated peptides as well as their low ionization efficiency often result in signal suppression when subjected to MS analysis.[10] Therefore, the development of a fast and specific protocol to enrich glycopeptides prior to MS becomes indispensable.

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Several methods have been developed for glycopeptide enrichment, i.e., lectins,[11−13] size exclusion,[14,15] hydrazide chemistry,[16−18] boronic acid affinity,[19−21] and hydrophilic interaction chromatography (HILIC).[22−27] Among them, HILIC-based methods have gained increasing popularity, which could not only provide good reproducibility, low bias, and convenience to automate but also avoid irreversible alterations of peptides, although the major deficiency of such is the limited selectivity caused by the coeluting nonglycosylated peptides. Recently, Liang et al.[28,29] synthesized a kind of saccharide-based hydrophilic matrix, named "click maltose" and applied it into the isolation of glycopeptides. Because of the enhanced hydrogen-bonding interaction generated by the flexible maltose chain between matrix and glycans of glycopeptides, the selectivity and glycosylation heterogeneity coverage was improved effectively, by which more than 140 glycopeptides covering all the five glycosylation sites of human serum α1-acid glycoprotein, were captured.

To determine N-linked glycosylation sites by MS, an efficient deglycosylation process is essential. Peptide-N-glycosidase F (PNGase F) is one of the most widely used glycosidases for liberating N-linked glycans, leading the deamidation of asparagine residue to aspartic acid simultaneously. However, the traditional deglycosylation protocol by PNGase F is usually performed in solution, which suffers from drawbacks, such as long incubation time, low deglycosylation yields, manual manipulation, and probably spontaneous nonenzymatic deamidation of the asparagine residue, caused by high temperature and pH, which would significantly affect the accuracy of the N-linked glycosylation site determination.[30] As a promising alternative, on-column deglycosylation with immobilized enzymatic reactors has drawn much attention. Novotny et al.[31] prepared a monolithic reactor with immobilized PNGase F to remove N-glycans from small- and medium-sized glycoproteins. Svec et al.[32] fabricated another two kinds of monolith based PNGase F-immobilized enzymatic reactors (IMERs), enabling the effective deglycosylation of even large glycoproteins. Later, an on-chip silica PNGase F-IMER was developed and integrated with other functional chips to perform glycoprotein deglycosylation, protein removal, glycan capture, and identification.[33] However, to the best of our knowledge, no applications of PNGase F-IMER for peptide deglycosylation and integration with glycopeptide enrichment have been exploited for N-linked glycosylation site profiling.

In this paper, to minimize the undesirable sample transfers, resultant sample loss, and rather long operation time, an integrated sample pretreatment system, involving glycopeptide enrichment by a click maltose HILIC column, buffer exchange by a strong cation exchange (SCX) precolumn, and deglycosylation by a PNGase F-IMER, was established for N-linked glycosylation site profiling. Through the application in the analysis of standard glycoproteins and the soluble fraction of rat brain extracts, our results demonstrate that such an online pretreatment system will provide an alternative method for large scale N-linked glycosylation site profiling.

EXPERIMENTAL SECTION

Reagents and Materials. Trypsin (bovine pancreas), IgG (human serum), avidin (egg white), urea (99.5%), and formic acid (FA) were ordered from Sigma-Aldrich (St. Louis, MO). Bovine serum albumin (BSA, bovine serum) was obtained from Sino-American Biotec (Luoyang, China). PNGase F was bought from New England Biolabs (Ipswich, MA). Dithiothreitol (DTT) and iodoacetamide (IAA) were from Acros (Morris Plains, NJ). Acetonitrile (ACN, HPLC grade) was ordered from Merck (Darmstadt, Germany). Water was purified by a Milli-Q system (Millipore, Milford, MA).

Fused-silica capillaries (150 μm i.d. × 375 μm o.d.) were obtained from Sino Sumtech (Handan, China). "Click maltose" matrixes (5 μm, 100 Å) were kindly donated by Prof. Xinmiao Liang (Dalian Institute of Chemical Physics, Chinese Academy of Science, Dalian, China). Strong cation exchange resins (5 μm, 500 Å) and acrylic polymer particles with amino groups (5 μm, 1000 Å) were obtained from Shenzhen Nanomicro Technology (Shenzhen, China). C18 aQ beads (5 μm, 120 Å) were ordered from Michrom BioResources (Auburn, CA).

Sample Preparation. The rat brain tissue was cleaned with water to remove possible contaminants, cut into small pieces, washed with PBS to remove blood, and then homogenized in buffer composed of 2 M NaCl and 1% (v/v) protease inhibitor complex cocktail in an ice bath. The resulting homogenate was centrifuged at 12 000 *g* for 40 min at 4 °C. Then, the supernatant was collected, and the protein concentration was measured by the Bradford assay.

IgG, avidin, BSA, and proteins extracted from rat brain were, respectively, dissolved in 8 M urea and then reduced in 10 mM DTT for 1 h at 56 °C. Subsequently, cysteines were alkylated in 20 mM IAA for 40 min at room temperature in the dark, followed by the dilution with 50 mM NH_4HCO_3 (pH 8.0), to decrease the urea concentration below 1 M. Finally, trypsin was added into the protein solution at an enzyme/substrate ratio (m/m) of 1:30 and incubated for 16 h at 37 °C. After the addition of 2 μL of formic acid to terminate the reaction, tryptic digests were desalted by homemade C18 precolumn and dried at low temperature using a Speed Vac Concentrator (Thermo-Fisher, San Jose, CA). All samples were stored at −20 °C before usage.

Glycopeptide Enrichment by Click Maltose Column. The HILIC column (300 μm i.d. × 5 cm) was in-house packed with click maltose matrixes. The enrichment procedure followed the protocol performed with a click maltose solid phase extraction (SPE)[29] but with some modifications. Briefly, the lyophilized tryptic digests of standard glycoproteins were redissolved in ACN−H_2O−FA (70:30:0.1) and then loaded onto the equilibrated column. At the flow rate of 12 μL/min, nonglycopeptides were removed by rinsing the column with ACN−H_2O−FA (70:30:0.1) for 15 min, and the glycopeptides were eluted with ACN−H_2O−FA (60:40:0.1) for another 15 min. All of the percentages refer to volume ratios unless otherwise specified.

For the offline evaluation of the click maltose HILIC column, fractions of glycopeptides were manually collected and subjected to matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF MS). For online analysis with the integrated pretreatment system, enriched glycopeptides were then trapped into a homemade SCX precolumn (150 μm i.d. × 2 cm), and further deglycosylated by PNGase F immobilized enzymatic reactor.

Preparation of PNGase F-IMER. Acrylic polymer particles with amino groups were packed into a capillary (150 μm i.d. × 8 cm). Subsequently, 25% glutaraldehyde in 0.1 M phosphate buffer (pH 8.0) was continuously flushed into the column for 6 h using a syringe pump (Baoding Longer Precision Pump, Baoding, China). Then 25 000 U/mL PNGase F in 0.1 M phosphate buffer (pH 8.0) containing 5 mg/mL sodium cyanoborohydride ($NaCNBH_3$) was continuously pumped into the column for 24 h at 4 °C. Finally, the residual aldehyde groups on the particles

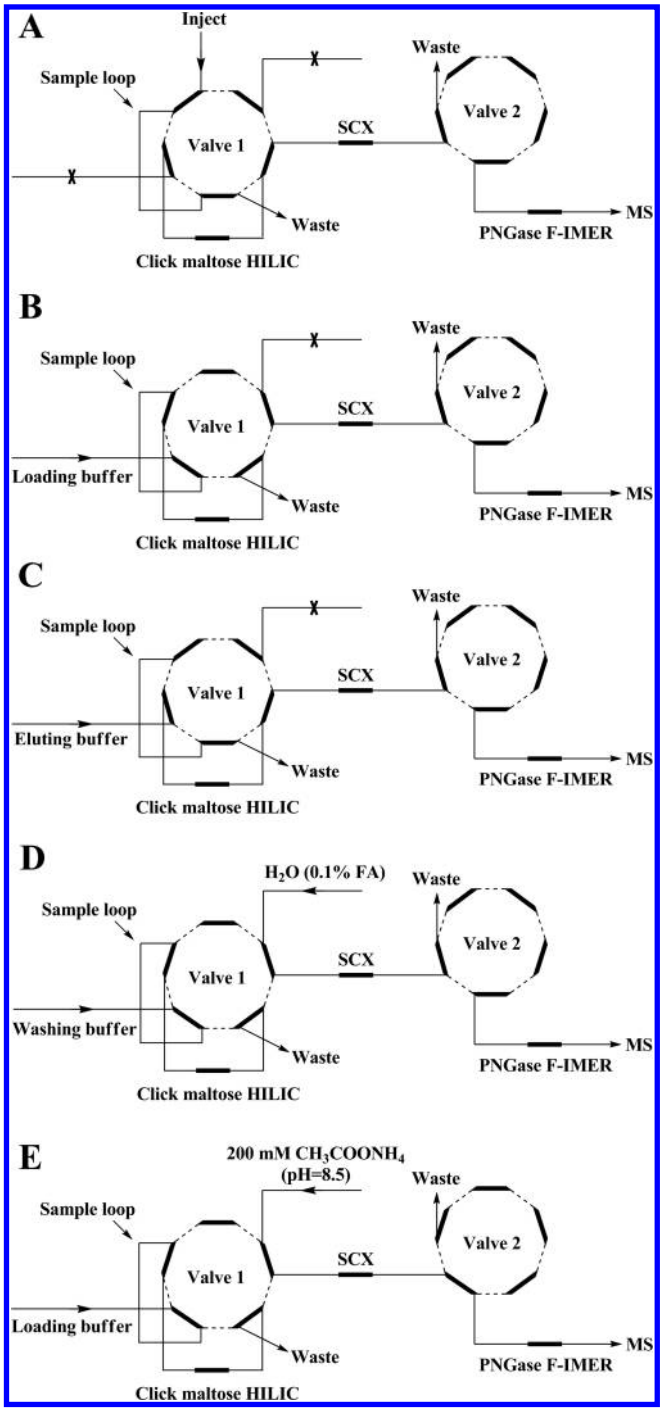

Figure 1. Flow diagram of the integrated sample pretreatment system for N-linked glycosylation site profiling.

were shielded by purging with 1 M Tris-HCl (pH 8.0) for 2 h, and the nonspecifically adsorbed PNGase F was removed by flushing with 20% acetonitrile in 0.1 M phosphate buffer (pH 8.0) for 4 h.

Peptide Deglycosylation. For the offline evaluation of PNGase F-IMER, 1 mg/mL standard glycoprotein digests in 50 mM CH_3COONH_4 (pH 8.5) were continuously pumped through the microreactor at the flow rate of 1 μL/min. The eluates were collected and then analyzed by MALDI-TOF MS.

For online deglycosylation, 200 mM CH_3COONH_4 (pH 8.5) was used to deliver enriched glycopeptides into the microreactor at a flow rate of 1 μL/min, and the resulting products were subjected to MALDI-TOF MS or nanoRPLC-ESI-MS/MS analysis. Unless otherwise specified, the peptide deglycosylation by IMER was carried out at room temperature.

For comparison, in-solution deglycosylation was performed by adding PNGase F (25 000 U/mL) into a solution, consisting of 1 mg/mL digest in 50 mM CH_3COONH_4 (pH 8.5), at an enzyme/substrate ratio of 1000 units/mg. After incubation at 37 °C for 16 h, the resulting peptides were analyzed by MALDI-TOF MS.

Online Glycopeptide Enrichment, Buffer Exchange, and Deglycosylation. The integrated system constructed on the UltiMate 3000 pump and flow manager (Dionex, Sunnyvale, CA) for glycopeptide pretreatment was shown in Figure 1, which consisted of a click maltose HILIC column for glycopeptide enrichment, an SCX precolumn for buffer exchange, and a PNGase F-IMER for N-linked glycan cleavage.

The tryptic digests were first injected into the sample loop (Figure 1A) and then flushed by the loading buffer, composed of 70% ACN with 0.1% FA, into the HILIC column at the flow rate of 12 μL/min for 15 min, to remove most nonglycosylated peptides (Figure 1B). After valve 1 switching, the captured glycopeptides were eluted into the SCX precolumn by rinsing the HILIC with the eluting buffer, composed of 60% ACN with 0.1% FA, at the same flow rate for another 15 min (Figure 1C). Subsequently, the remaining acetonitrile on the SCX column was flushed with 0.1% FA at the flow rate of 1 μL/min for 10 min, and the HILIC column was washed with ACN−H_2O−FA (10:90:0.1), simultaneously (Figure 1D). Finally, after valve 2 switching, 200 mM CH_3COONH_4 (pH 8.5) was pumped into the SCX at the flow rate of 1 μL/min for 20 min, by which the eluted peptides were delivered into PNGase F-IMER for online deglycosylation. Simultaneously, the HILIC column could be equilibrated with the initial loading buffer for the next autorun (Figure 1E). The collected peptides from IMER were further analyzed by MALDI-TOF MS.

In contrast to that of standard proteins, N-linked glycosylation site profiling of rat brain proteins was performed by rinsing the HILIC column with ACN−H_2O−FA (75:25:0.1) and eluting glycopeptides with ACN−H_2O−FA (50:50:0.1). Other conditions were the same as mentioned above. The obtained peptides were further analyzed by nanoRPLC-ESI-MS/MS.

MS Identification and Data Analysis. MALDI-TOF MS was performed on Ultraflex III TOF/TOF (Bruker Daltonics, Bremen, Germany). α-Cyano-4-hydroxycinnamic acid (CHCA) matrix solution (7 mg/mL) was prepared in ACN−H_2O−TFA (60:40:0.1). Equivalent amounts of the sample and CHCA were sequentially dropped onto the MALDI plate for MS analysis. Spectra were obtained in positive ionization mode using reflector detection.

The deglycosylated peptides enriched from rat brain digests were analyzed by the nanoRPLC-ESI-MS/MS system, consisting of a quaternary surveyor MS pump (Thermo Fisher, San Jose, CA) and an LTQ-Orbitrap mass spectrometer (Thermo Fisher). The lyophilized sample was dissolved in 5 μL of 0.1% FA solution, and 1 μL was loaded onto a homemade capillary separation column (75 μm i.d. × 14 cm). Mobile phase A (0.1% FA in H_2O) and B (0.1% FA in ACN) were used to establish the 110 min gradient, comprised of 2 min of 0−3% B, then 80 min of 3−35% B, followed by 18 min of 35−80% B, and finally maintained at 80% B for 10 min, with the flow rate at 200 nL/min.

The LTQ-Orbitrap mass spectrometer instrument was operated in positive mode with a 1.8 kV applied spray voltage. The

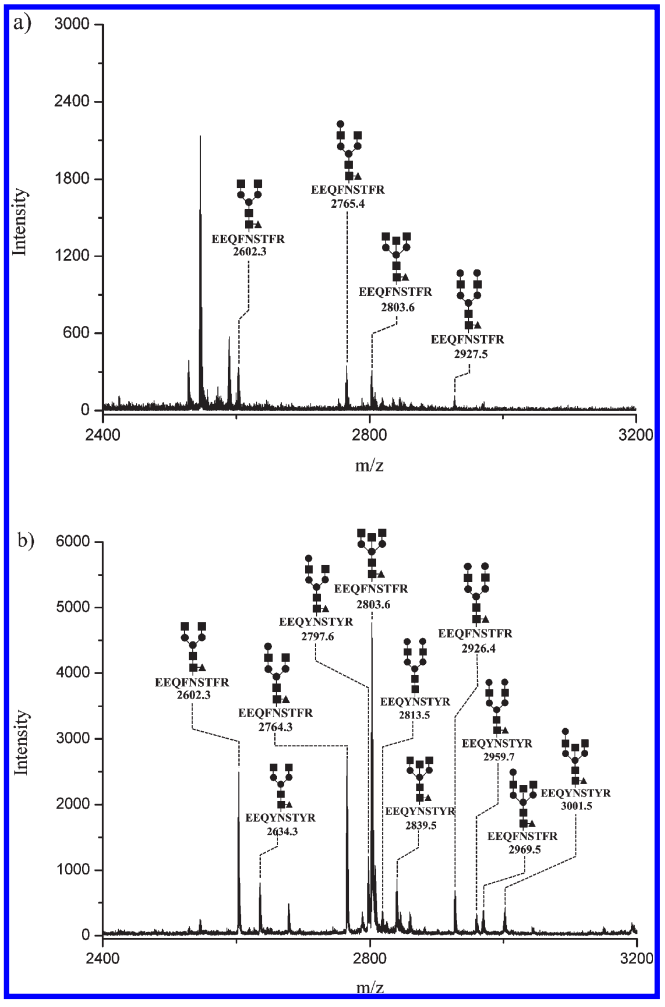

Figure 2. MALDI-TOF mass spectra of the IgG digest (50 ng/μL) (a) before and (b) after enrichment with the click maltose HILIC column. The peaks of glycosylated peptides are marked according to their mass charge ratios and structures. Symbols: ■, *N*-acetylglucosamine; ●, mannose or galactose; ▲, fucose.

temperature of the ion transfer capillary was set at 200 °C. One microscan was set for each MS and MS/MS scan. All MS and MS/MS spectra were acquired in the data dependent mode. A full scan MS acquired from m/z 400 to 2000 was followed by six data dependent MS/MS events. The dynamic exclusion function was set as follows: repeat count, 1; repeat duration, 30 s; exclusion duration, 180 s. The normalized collision energy for MS/MS scanning was 35%.

Raw MS data was searched against the International Protein Index (IPI) Rat v3.26 database using SEQUEST algorithms incorporated in BioWorks software (version 3.3.1), and reversed sequences were appended to the database for estimating the false discovery rate (FDR). The parameters for the SEQUEST search were as follows: enzyme, fullTrypsin; missed cleavages, two; fixed modifications, carboxyamidomethylation (C); variable modifications, oxidation (M) and deamidation (N); peptide tolerance, 10 ppm; MS/MS tolerance, 1.0 Da. The resulting .out files were input into a software tool BuildSummary to prevent redundancy in protein identifications. The ΔCn value was set as 0.1, and Xcorr values were adjusted to ensure the FDR of peptide identification is less than 1%. If there was any peptide that was assigned to several independent accessions, the above independent accessions were sorted to a protein group corresponding to the peptide.[34] Since N-glycosylation often occurs at a consensus motif of N-!P-S/T, the remaining peptide sequences were additionally filtered to remove nonmotif containing peptides. The identified proteins were further verified and categorized by the Swiss-Prot Database.

RESULTS AND DISCUSSION

The goal of this work is to provide an efficient integrated pretreatment system for N-linked glycosylation site profiling including glycopeptide enrichment by click maltose HILIC and deglycosylation by PNGase F-IMER via an SCX precolumn to ensure the buffer compatibility.

Evaluation of Click Maltose HILIC Column. As a novel HILIC matrix, click maltose matrix has been successfully applied for glycopeptide enrichment, with improved selectivity and glycosylation heterogeneity coverage.[29] In comparison to the packed SPE tip applied in previous work, the chromatographic column was more convenient to operate and compatible with an online sample pretreatment system.

To evaluate the performance of the click maltose HILIC column, IgG, which contains one N-linked glycosylation site in each of its variants (IgG1 and IgG2), was utilized. Tryptic digest (50 ng/μL) dissolved in ACN−H_2O−FA (70:30:0.1) was loaded onto the column, and the captured glycopeptides were analyzed by MALDI-TOF MS. In the spectrum of the native digest, only four weak signals matching to the glycosylated peptides could be detected (Figure 2a). After enrichment with the click maltose HILIC, the above-mentioned four glycopeptides were observed with improved signal intensity and an additional seven glycosylated peptides were identified (Figure 2b), demonstrating that such a HILIC column was applicable and effective for enriching glycopeptides.

Evaluation of PNGase F-IMER. In our experiments, an enzymatic microreactor was prepared, by immobilizing the PNGase F on polymer particles, and the performance was evaluated with IgG as the sample.

Slightly different from the published protocol on PNGase F-IMER evaluation,[31,32] herein the deglycosylation was performed at the peptide level. When 1 mg/mL IgG digest in 50 mM CH_3COONH_4 (pH 8.5) was directly analyzed by MALDI-TOF MS, various peaks in the range between m/z 2400 and 3200, assigned to the diverse glycopeptides of IgG, were observed in the spectrum (Figure 3a). After treatment with PNGase F-IMER at the flow rate of 1 μL/min, corresponding to a residence time of 1.4 min in the microreactor, the signals of glycosylated peptides in the high m/z range almost disappeared, while the peaks of deglycosylated glycopeptides at m/z 1158.8 and 1190.8, representing EEQFN#STFR and EEQYN#STYR (in which N# denotes the N-linked glycosylation site), were evidently detected (Figure 3b), which demonstrated the efficient enzymatic cleavage of the N-linked peptides from the sugar moiety. For comparison, in-solution deglycosylation of IgG digest with an incubation time of 16 h was also performed (Figure 3c). In both cases, similar mass spectral profiling was obtained, in which most of glycopeptides were efficiently deglycosylated, except that one and two glycosylated peptide signals (with mass charge ratios and structures marked in insets of Figure 3b,c) still remained after deglycosylation by PNGase F-IMER and soluble PNGase F, respectively. The results indicated that the liberation of the suger

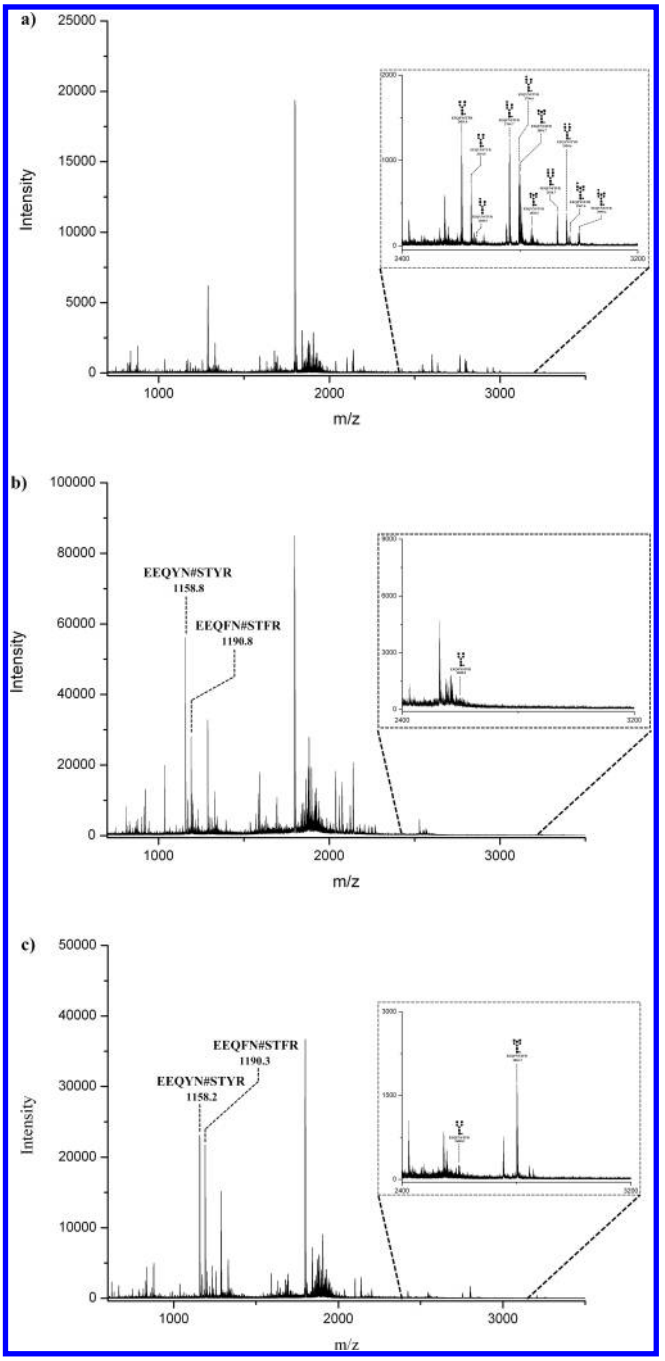

Figure 3. MALDI-TOF mass spectra of IgG digests (1 mg/mL) (a) before, (b) after treatment with PNGase F-IMER (residence time, 1.4 min), and (c) after treatment with soluble PNGase F (incubation time, 16 h). The insets show peaks in the range between m/z 2400 and 3200. The peaks of deglycosylated glycopeptides are marked according to their sequences and mass charge ratios. The peaks of glycosylated peptides are labled with their mass charge ratios and structures. Symbols: ■, *N*-acetylglucosamine, ●, mannose or galactose; ▲, fucose.

moiety from glycosylated peptides by IMER was comparable and even superior to that achieved with PNGase F in solution. Importantly, the reaction time was shortened to 1/685 (1.4 min vs 16 h) after the PNGase F immobilization, demonstrating that the polymer particle based microreactor was suitable for effective glycopeptide deglycosylation with a high speed. The performance of such a microreactor was further evaluated by avidin, another N-glycosylated protein with the site at Asn_{41}, and the applicability of the PNGase F-IMER for other glycoproteins was validated (see Figure S-1 in the Supporting Information).

Analysis of Glycopeptides with Integrated System. Since the glycopeptide fractions from HILIC are of high organic modifier concentration and low pH value, incompatible with online deglycosylation by PNGase F-IMER, herein an SCX precolumn was applied to the buffer exchange for HILIC eluates to achieve online hyphenation with PNGase F-IMER.

When both valves were switched at the position shown in Figure 1C, the eluates from HILIC with the captured glycopeptides could pass through the connected SCX precolumn, on which 86% of the glycopeptides were trapped (see Figure S-2 in the Supporting Information). After the SCX column was washed with 0.1% FA, acetonitrile was removed without further contact with PNGase F-IMER (Figure 1D). Subsequently, the trapped glycopeptides were eluted into IMER with 200 mM CH_3COONH_4 (pH 8.5) for online deglycosylation (Figure 1E). The increased salt concentration was beneficial to decrease the peptide residual on SCX and proved to have no effect on the enzymatic activity of PNGase F-IMER (see Figure S-3 in the Supporting Information). Therefore, with the SCX precolumn setting in between, the establishment of an integrated system with the online combination of click maltose HILIC and PNGase F-IMER became possible. The undesired loss of glycopeptides by SCX might be attributed to the diverse properties of glycopeptides and the high concentration of acetonitrile in the eluting buffer. Further effort to improve the glycopeptide trap ability of the SCX precolumn is underway by decreasing the acetonitrile concentration and improving the binding capacity of the SCX precolumn.

In addition, the total sample pretreatment time by our system was 1 h, which was ~1/17 of that required by the offline HILIC based methods. When compared with the classical hydrazide chemistry, involving multiple chemical reactions and resin clean-up steps, the time could be shortened to at least 1/28.[18] Importantly, the whole pretreatment procedure could be performed automatically via the valve changing program, without the risk of sample loss and contamination.

With the integrated system, the tryptic digests of avidin and IgG (1 μg, dissolved in 70% ACN containing 0.1% FA) were analyzed, respectively. As shown in Figure 4a,b, the peak at m/z 1837.2, representing the N-linked deglycosylated glycopeptide WTNDLGSN#MTIGAVNSR of avidin, as well as the peaks at m/z 1158.6 and 1190.6 assigned to EEQYN#STYR and EEQFN#STFR of IgG, respectively, were identified by MALDI-TOF MS with a high S/N (821, 481, and 83), suggesting that the integrated system could enable the efficient isolation and deglycosylation of glycosylated peptides. When the digests of both avidin and IgG were mixed at the ratio (m/m) of 1:1, all the three deglycosylated glycopeptides were detected with good specificity (Figure 4c), which indicated that the system was applicable for analyzing glycopeptide mixture with various glycosylation sites.

The selectivity of this integrated system was further evaluated by detecting deglycosylated glycopeptides from the digest mixture of avidin and BSA, at a ratio (m/m) of 1:10 and 1:50, respectively, with the total amount fixed at 1 μg. When BSA was 10 times more than avidin, the signal of m/z 1837.4 could be selectively detected (Figure 5a). When BSA was 50 times more than avidin, where the amount of avidin was estimated to be 20 ng, the signal of deglycosylated glycopeptides could still be observed with a S/N of 48 (Figure 5b). All these results

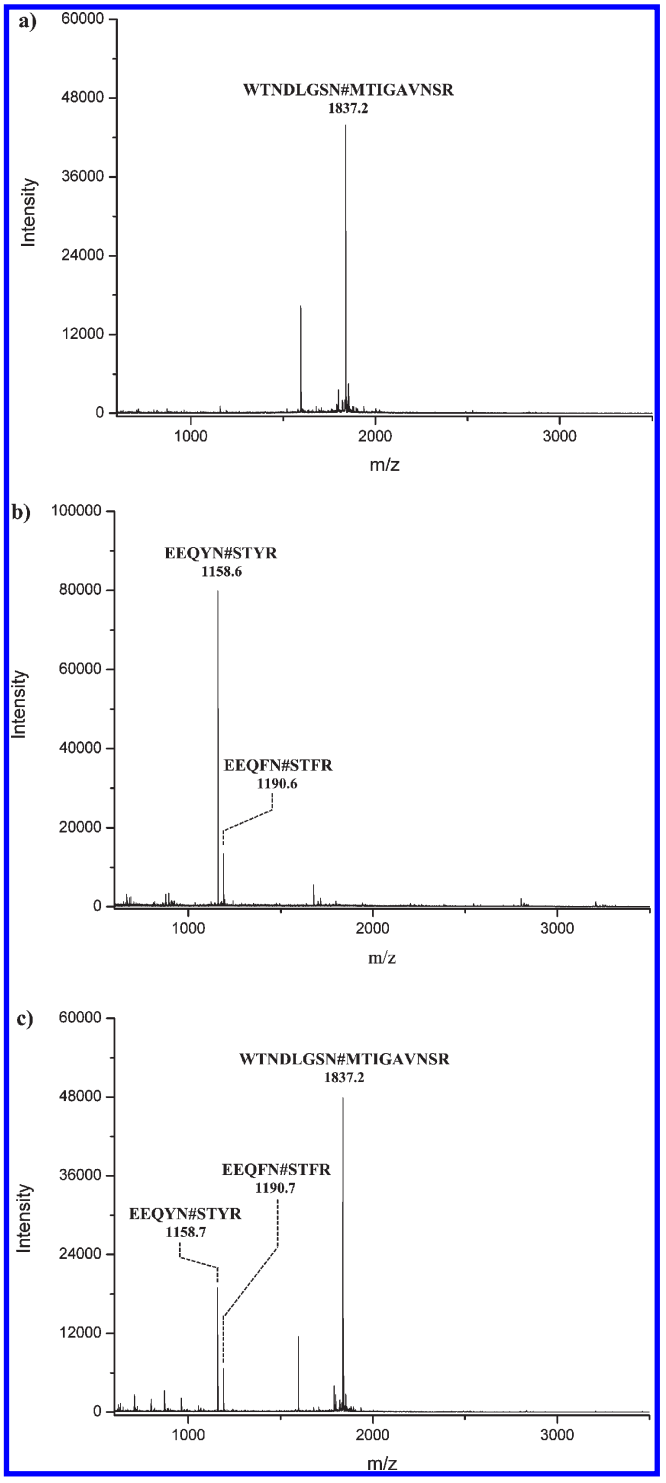

Figure 4. MALDI-TOF mass spectra of deglycosylated glycopeptides analyzed by the integrated system from (a) avidin digest, (b) IgG digest, and (c) digest mixture of avidin and IgG at the ratio (m/m) of 1:1. The peaks of deglycosylated glycopeptides are marked according to their sequences and mass charge ratios.

demonstrated the specific detecting ability of the integrated system in N-linked glycosylation sites determination.

Moreover, the reproducibility and sensitivity of N-linked glycopeptide identification were also studied with the integrated system. Through the treatment of the avidin digest (1 μg) three

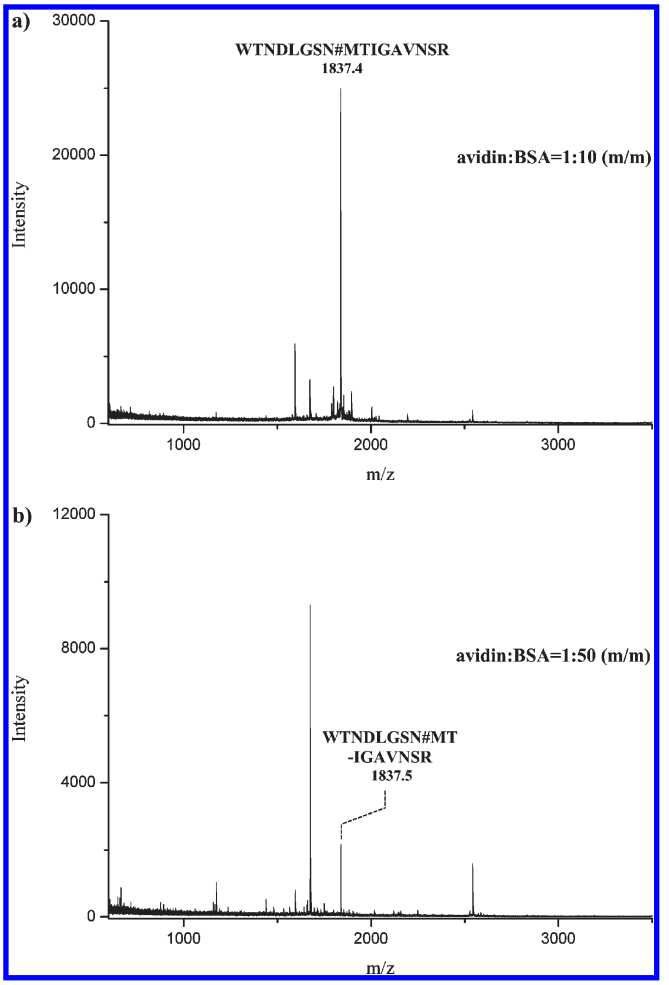

Figure 5. MALDI-TOF mass spectra of deglycosylated glycopeptides enriched by the integrated system from the digest mixture of avidin and BSA at the ratio (m/m) of (a) 1:10 and (b) 1:50. The peaks of deglycosylated glycopeptides are marked according to their sequences and mass charge ratios.

times, followed by the analysis of the eluates with MALDI-TOF MS, it could be seen that the peptide profiling in three consecutive runs were of good reproducibility and the signal intensity ratio of deglycosylated peptide m/z 1837 and non-glycopeptide m/z 1594 had the relative standard deviation (RSD) of 8% (see Figure S-4 in the Supporting Information). Even for 5 fmol of avidin digest, the deglycosylated glycopeptide was also detected with a S/N of 7.4 by MALDI-TOF MS (see Figure S-5 in the Supporting Information). All these results demonstrated that our developed integrated system was of good reproducibility and high sensitivity for the identification of N-linked glycopeptides.

Identification of N-Linked Glycoproteins in Rat Brain. To evaluate the applicability of our integrated system for complex samples, 6 μg of tryptic digest of the soluble fraction extracted from rat brain was pretreated by the system, followed by further nanoRPLC-ESI-MS/MS analysis. In total, 437 unique peptides mapped to 223 nonredundant protein groups were identified, of which 120 protein groups belonged to N-linked glycoprotein groups and 193 peptides could be assigned to N-linked glycopeptides (see Table S-1 in the Supporting Information). Most glycopeptides are of a single consensus site, except three

sequences with double glycosites. Among all the N-linked glycosylation sites identified, 9 sites (4.6%) have been reported by previous references according to the information in the Swiss-Prot Database, 126 sites (64.3%) were annotated as potential, and 61 sites (31.1%) were newly discovered in this study. Although recently, various enrichment methods and hyphenated techniques have been attempted to profile the N-glycosylation information of proteins from the real biological samples, several good identification results were obtained, and the required sample amounts in previous work were relatively high, i.e., 270 μg[21] or even 1 mg.[27] With the similar number of the identified glycosylated peptides and proteins, the amount of the sample by our protocol was greatly decreased, which might be attributed to the high sensitivity and low sample loss obtained by the integrated pretreatment system.

CONCLUSIONS

An integrated pretreatment system for N-linked glycosylation site profiling was established, in which glycopeptides were enriched by a click maltose HILIC column and deglycosylated by a PNGase F-IMER via an SCX precolumn in between to improve the buffer compatibility, rendering fast and automatic operation with low sample loss or contamination. Compared with the conventional offline method, the total pretreatment time was shortened to 1 h, and the detection limit was reduced to as low as 5 fmol. Moreover, the increased selectivity and glycosylation heterogeneity coverage of click maltose HILIC as well as the probable avoidance of the spontaneous nonenzymatic deamidation of asparagine residue by PNGase F-IMER enable the large-scale and accurate profiling of N-linked glycosylation sites in real samples. Such an integrated system could also be further online coupled with peptide separation and identification systems to achieve high-throughput and high-sensitivity analysis. When combined with the quantitative proteomic method, differential identification and quantification of disease related glycoproteins would be anticipated.

ASSOCIATED CONTENT

Supporting Information. Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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