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Online Proteolysis and Glycopeptide Enrichment with Thermoresponsive Porous Polymer Membrane Reactors for Nanoflow Liquid Chromatography-Tandem Mass Spectrometry

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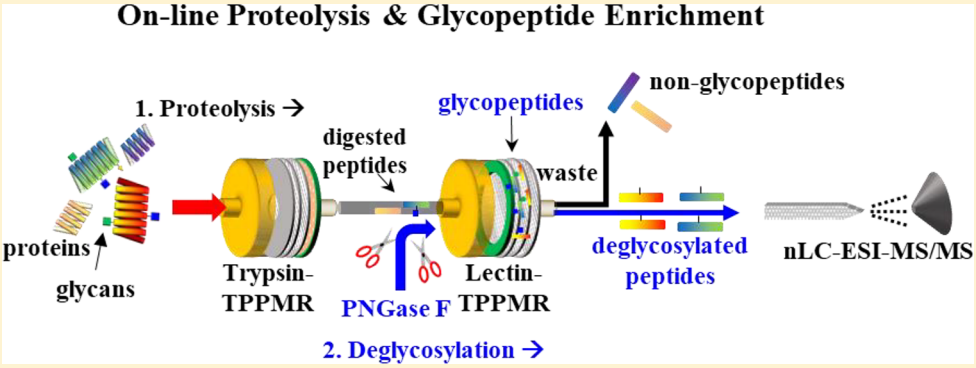
Supporting Information



ABSTRACT: In this Article, we have reported a fully automated online method to carry out proteolysis and glycopeptide enrichment in sequence for nanoflow liquid chromatography-tandem mass spectrometry (nLC-ESI-MS/MS) analysis. By implementing two serial thermoresponsive porous polymer membrane reactors (TPPMRs), in which the TPPM could be immobilized either with trypsin for proteolysis or with lectins for glycopeptide enrichment, the entire pretreatment procedure can be performed online in about an hour. The TPPM was fabricated by coating polystyrene-maleic anhydride-*N*-isopropylacrylamide (PS-MAn-PNIPAm), which was synthesized by reversible addition−fragmentation chain transfer polymerization, on a Nylon sheet. Because of the thermoresponsive nature of PNIPAm, it formed micelle cavities and changed its morphology at elevated temperatures, resulting in enhanced interactions between the enzyme or lectins and the proteins/peptides flowing through the membrane. The performances of the TPPMs were evaluated by varying the temperature conditions and the amount of standard proteins, showing that both proteolysis and glycopeptide enrichment with online deglycosylation were highly efficient at 37 °C. The developed online serial TPPMRs-nLC-ESI-MS/MS method was applied to the human plasma sample (1.5 μL) and a total of 262 *N*-glycopeptides could be identified from 155 glycoproteins. Thus, the present work demonstrates a fully automated high speed analytical protocol for online proteolysis and glycopeptide enrichment, which is extremely useful for analyzing small amounts of the proteome samples.

Glycosylation is a post-translational modification of proteins that occurs either in the endoplasmic reticulum (ER) or the Golgi apparatus in cells with the assistance of several enzymes. Glycosylated proteins account for nearly half of total human proteins[1] and play important roles in protein folding, cell-to-cell interaction, and immune response at the surface of the cell membrane.[2−4] The most common form of glycoproteins is the *N*-linked glycoprotein, which is produced by the *N*-glycosidic linkage of glycan with the asparagine residue that is present as a part of Asn-X-Ser/Thr (where X is any amino acid except proline).[5] Since *N*-linked glycoproteins are known as diagnostic markers of prostate and liver cancers,[1,6,7] and are reported to be strongly related to neurodegenerative diseases,[8,9] an accurate analysis of *N*-linked glycoproteins is important in the development of biomarkers to study and diagnose various diseases.

Analysis of *N*-linked glycoproteins has been empowered by mass spectrometry (MS), which provides an accurate determination of the glycosylation sites by tandem mass spectrometric analysis such as collision-induced dissociation (CID), electron-capture dissociation, and electron-transfer dissociation.[10,11]

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While MS-based methods have continuously evolved, it is still challenging to analyze glycopeptides because peptides containing glycans are present in relatively small amounts and are difficult to ionize. Therefore, the enrichment of glycopeptides is the key to improving their analysis.[12,13] Several methods have been developed to isolate or enrich glycopeptides prior to MS analysis. While immobilization of antibodies on the hydrazine resin is a reproducible and relatively inexpensive method, it nevertheless takes a long time.[14,15] The solid phase extraction method, based on functionalized magnetic nanoparticles,[16] is fast and automatable but the performance depends on the quality of the nanomaterials. Hydrophilic interaction chromatography[17] can provide a selective elution of glycopeptides, but requires proteolysis to be conducted beforehand. While lectin-affinity chromatography[18−20] demonstrates a good capability to accommodate glycans attached to proteins/peptides, some nonspecific bindings are also present. Owing to the high binding capacity of lectins to the glycans,[12,21] several studies have been conducted with the lectin-based enrichment process, which is based on the principle of immobilization of lectins on either a microarray,[22] magnetic beads,[23] or resin material.[24] Despite the simplicity of glycopeptide enrichment, these techniques generally require off-line operations and cannot avoid several weaknesses such as sample loss during purification steps, long process time, and the limitation to small amounts of the sample. To overcome these shortcomings, online enrichment and purification methods that can be fully automated from sample loading to analysis in sequence without a stop, are required. Several efforts have been performed to carry out the online digestion of proteins,[25,26] or online enrichment of glycopeptides from predigested peptide mixtures.[27] An online digestion and solid-phase extraction of glycopeptides using graphitized carbon sorbents was demonstrated with a model protein (ribonuclease B).[28] However, it has not as yet been achieved with a comprehensive online procedure that includes both digestion and glycopeptide-specific enrichment for human proteome research.

This study introduces a fully automated online method to carry out proteolysis followed by enrichment of glycopeptides in sequence for nanoflow liquid chromatography-electrospray ionization-tandem MS (nLC-ESI-MS/MS) analysis. In an earlier study, we introduced an online proteolysis method based on utilizing the polystyrene-maleic anhydride (PS-MAn) porous polymer membrane reactor.[29] However, because of the relatively large pore size, there was a limit to the improvements in efficiency of the immobilization of trypsin. The method proposed in this study is based on two serial thermoresponsive porous polymer membrane reactors (TPPMRs), in which micelle cavities can be controlled by incorporating *N*-isopropylacrylamide (NIPAm) to PS-MAn system so that the PS-MAn-NIPAm membranes are immobilized with trypsin on one TPPMR and with lectin mixtures on the other one. The thermoresponsive porous polymer membrane (TPPM) was fabricated by synthesizing PS-MAn-NIPAm via reversible addition−fragmentation chain transfer (RAFT) polymerization followed by coating on a nylon membrane. The efficiencies of the TPPMRs were evaluated for proteolysis using bovine serum albumin (BSA) in the trypsin-TPPMR and for glycopeptide enrichment using the α-1-acid glycoprotein (AGP) in lectins-TPPMR by varying the temperature and the loading amount of proteins. The efficiency of the online TPPMRs-nLC-ESI-MS/MS system was compared with the off-line enrichment of AGP glycopeptides, and then it was applied to a human blood plasma proteome sample to demonstrate the fully automated enrichment of *N*-glycopeptides.

EXPERIMENTAL SECTION

Materials and Reagents. Styrene (S), maleic anhydride (MAn), *N*-isopropylacrylamide (NIPAm), and 2-(dodecylthiocarbonothioylthio)-2-methylpropionic acid (DTMA) were purchased from Beijing InnoChem Science & Technology Co., Ltd. (Beijing, China). Tetrahydrofuran (THF), 1,4-dioxane, diethyl ether, dimethyl sulfoxide (DMSO), azobis(isobutyronitrile) (AIBN), and other chemicals were of analytical reagent grade purity and supplied by Beijing Chemical Corporation (Beijing, China). AIBN was purified before use. *N*-α-Benzoyl-L-arginine (BA) was obtained from Aladdin Reagents Industrial Co., Ltd. (Shanghai, China) and *N*-α-benzoyl-L-arginine ethyl ester (BAEE) was purchased from Acros Organics (Fair Lawn, NJ, USA). Bovine serum albumin (BSA), α-1 acid glycoprotein (AGP), concanavalin A (ConA), and wheat germ agglutinin (WGA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sequencing-grade trypsin and *N*-glycosidase F (PNGase F) were obtained from Promega Corp. (Madison, WI, USA). HPLC grade water and acetonitrile were from J.T. Baker (Phillipsburg, NJ, USA). Fused silica capillary tubes (75, 100, and 200 μm i.d., and 360 μm o.d. for all) were purchased from Polymicro Technology LCC (Phoenix, AZ, USA).

Human Plasma Samples for Online Proteolysis and Glycopeptide Enrichment. Human plasma samples were obtained with the approval of the Institutional Review Board (IRB) from the Severance Hospital Gene Bank (Seoul, Korea). A volume of 50 μL of the pooled human plasma samples (from 5 healthy adults) were treated with the PureExtract Albumin/IgG Depletion kit from Merck Millipore (Billerica, MA, USA) to remove the high abundance proteins. Throughout the experiments, the albumin/IgG depleted pooled plasma sample was utilized. The resulting depleted plasma sample (~1 mL) was concentrated to 100 μL by using Amicon Ultra-0.5 mL centrifugal filters (nominal molecular weight limit = 3 kDa) from Merck Millipore. Protein concentration was measured as 3.32 mg/mL by the Bradford assay and 3 μL (approximately 10 μg and equivalent to 1.5 μL of the original plasma) of proteome was injected into the online serial TPPMRs-nLC-ESI-MS/MS. In order to compare with online proteolysis and glycopeptide enrichment, protein digestion, and glycopeptide enrichment of 3 μL of the depleted plasma sample were carried out by the solution method, as described in the Supporting Information. All of the samples injected into the TPPMR, including the human plasma and protein standards, were added with 10 mM dithiothreitol and denatured at 45 °C for 30 min prior to online analysis.

Preparation of TPPM. The copolymers of PS-MAn and PS-MAn-NIPAm were synthesized by RAFT polymerization (Figure S1). The structures of the copolymers were characterized with a model DMX-400 spectrometer from Bruker (Billerica, MA, USA) (Figure S2) and TENSOR-27 FT-IR spectrometer from Bruker Technology Co., Ltd. (Beijing, China) (Figure S3). The average molecular weights of the prepared copolymers were 36.8 kDa for PS-MAn and 40.8 kDa for PS-MAn-NIPAm, as measured by gel permeation chromatography.

TPPM was prepared by using the breath figure method,[30,31] as shown in Figure S4a. The nylon sheet (50 μm in pore size and 95 μm in thickness) was put in a humid environment (relative humidity ~90%). The PS-MAn-NIPAm solution dissolved in chloroform (30.0 mg/mL) was dripped on the nylon sheet

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Table 1. Sequence Coverage (%), the Number of Identified Peptides, the Number of Mis-cleaved Peptides from BSA by Using TPPMR, and the Comparison of the Digestion Efficiency under Different Temperatures Using Various Amounts of BSA

	sequence coverage (%)			number of identified peptides			number of mis-cleaved peptides		
amount of injected proteins (ng)	25 °C	37 °C	45 °C	25 °C	37 °C	45 °C	25 °C	37 °C	45 °C
10	6.8 ± 0.6	6.5 ± 0.4	6.8 ± 1.7	3 ± 1	3 ± 1	3 ± 1	1 ± 1	2 ± 1	1 ± 1
100	45.4 ± 3.1	67.4 ± 3.6	62.0 ± 1.9	33 ± 4	45 ± 4	41 ± 3	14 ± 2	21 ± 2	19 ± 1
500	62.1 ± 1.9	92.2 ± 1.3	78.9 ± 1.9	41 ± 3	83 ± 2	59 ± 5	23 ± 3	52 ± 3	39 ± 1
1000	75.7 ± 1.9	93.5 ± 0.9	87.7 ± 6.5	53 ± 2	85 ± 2	78 ± 2	32 ± 2	53 ± 2	45 ± 2

dropwise. After evaporation of all solvents under 90% humidity conditions, fabricated TPPM was examined with a model S-4800 scanning electron microscope (SEM) from Hitachi Co. (Tokyo, Japan). A relatively uniform distribution of pore sizes (737 ± 214 nm) was observed, which was suitable for the immobilization of the enzyme (Figure S4b).

Evaluation of TPPM for Enzyme Immobilization. The efficiency of TPPM for enzyme immobilization was evaluated by inserting the TPPM into a 6.5 mm i.d. precolumn filter assembly and washing with 50.0 mM of the Tris-HCl (pH 8.4) solution for 3 h. Subsequently, a mixture of 2.0 mg/mL trypsin and 50.0 mM benzamidine (dissolved in 50.0 mM Tris-HCl solution, pH 8.4) was delivered to the unit at a flow rate of 10 μL/min by varying the immobilization time, followed by rinsing with phosphate buffered saline (PBS) (pH 8.2). Enzymatic hydrolysis was evaluated by comparing the peak areas of BA and the hydrolyzed product of BAEE by a 1229 high performance capillary electrophoresis (HPCE) analyzer from the Beijing Institute of New Technology and Application (Beijing, China) using bare capillaries (75 μm I.D. × 70 cm, 55 cm effective) from the Yongnian Optical Fiber Factory (Hebei, China) at 25 °C. This was achieved by delivering 2.0 mM BAEE dissolved in 50.0 mM Tris-HCl solution (pH 7.5) into the filter unit at a flow rate of 30 μL/min for 10 min. The effluent was then collected for HPCE analysis. The effect of different temperatures, enzyme immobilization times, number of membrane layers, trypsin concentration, copolymers either with or without PNIPAm, and the ratio of PNIPAm/PS-MAn in the copolymers on the efficiency of hydrolysis were investigated in detail. From initial evaluations, it was found that the efficiency of enzymatic hydrolysis was enhanced at 37 °C (Figure S5a) with 24 h as the immobilization time (Figure S5b). Moreover, stacking two layers of TPPM (Figure S5c) was found to be much more efficient for enzyme immobilization as compared to using a single layer. Optimum concentration for trypsin for hydrolysis was 2.0 mg/mL (Figure S5d). The incorporation of PNIPAm on PS-MAn provided significant improvements in the hydrolysis efficiency under all temperature conditions (26, 37, and 45 °C), as compared to the polymers without PNIPAm (Figure S5e). Since PNIPAm chains on the porous copolymer curled to form micelle cavities at a high temperature, they were capable of confining the enzyme more efficiently, as illustrated in Figure S6. The results of the contact angle measurements are shown in Figure S7. The optimum ratio of PNIPAm to PS-MAn was determined to be 1:1 (Figure S5f).

Assembly of the TPPMR Module. Microscale TPPMR modules for online connection with nanoflow liquid chromatography (nLC)-ESI-MS/MS were prepared by inserting TPPMs into the PEEK precolumn filter unit (1.4 μL of original swept volume) from IDEX Health & Science, LCC (Oak Harbor, WA, USA). To fabricate a TPPMR for proteolysis (trypsin-TPPMR), two layers of TPPM were stacked with a polychlorotrifluoroethylene (PCTFE) ring (0.25 in. o.d., 0.188 in. i.d., and 0.062 in. thickness) and a regenerated cellulose membrane (10 kDa cutoff) from Merck Millipore, as shown in Figure S8a. Trypsin was immobilized by delivering 20 μg of sequencing-grade trypsin in 50 mM Tris-HCl (pH 8.4) buffer at 5 μL/min for 2.5 h using a Legato 110 syringe pump from KD Scientific (Hollistone, MA, USA) into the trypsin-TPPMR. For the lectin-immobilized TPPMR (lectin-TPPMR), three layers of TPPM were used to ensure enzyme immobilization and 3 mg/mL of the lectin mixtures (3:1 (w/w) ConA:WGA) were delivered overnight at 3 μL/min. Connection of TPPMR with the syringe pump was made with a fused silica capillary tube.

nLC-ESI-MS/MS Analysis. Glycopeptides enriched from the TPPMR systems were analyzed online by using two nLC-ESI-MS/MS, namely, a Dionex Ultimate 3000 RSLCnano liquid chromatography (LC) system with an autosampler coupled to an LTQ Velos ion trap mass spectrometer from Thermo Fischer Scientific (San Jose, CA, USA) and the model 1260 capillary LC system from Agilent Technologies (Waldbronn, Germany) with a Q Exactive hybrid quadrupole-orbitrap mass spectrometer from Thermo Fischer Scientific. An analytical column (15 cm × 75 μm i.d.) was prepared in the laboratory by packing 3.5 μm-sized XBridge Peptide BEH (ethylene-bridged hybrid) particles (130 Å) unpacked from a BEH column from Waters for nLC separation into a pulled tip capillary as described elsewhere.[32] A trap column was utilized prior to the analytical column connected with a PEEK microcross and it was packed with 3 μm-200 Å Magic C18AQ particles obtained from Michrom Bioresources Inc. (Auburn, CA, USA) for 3 cm in a 200 μm i.d. capillary tube of which one end was ended with a sol−gel frit. The gradient elution conditions are described in the Supporting Information.

Data analysis was carried out with the Proteome Discover Software (version 1.4.0.288) from Thermo-Finnigan against the UniprotKB human database (released July 2017) with the SEQUEST HT algorithm. The variable modification was set as the oxidation of methionine (+15.995 Da) and deamidated asparagine (+0.984 Da). During the database search, the mass tolerance values were set as 1.0 Da for the precursor ions and 0.8 Da for the product ions along with the thresholds: 0.1 for ΔCn score and 2.4, 2.7, and 3.7 for the minimum cross-correlation (Xcorr) values of +1, + 2, and +3 charged ions,[33] respectively, with the false discovery rate (FDR) of 0.01.[34]

RESULTS AND DISCUSSION

Digestion Efficiency of the TPPMR Module. The performances of TPPMRs for both proteolysis and glycopeptide enrichment were evaluated separately. The efficiency of proteolysis by the trypsin-immobilized TPPMR module was evaluated using BSA. At first, the inner volume of the trypsin-immobilized TPPMR was reduced to 0.1 μL by replacing the PCFTE ring (shown in Figure S8a) with a Teflon spacer (3 mm i.d., 100 μm thickness) to enhance the enzymatic reaction. Furthermore, the RC membrane underneath the TPPMs was removed as shown in the Figure S8b. The online digestion

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efficiency of trypsin-TPPMR was examined with BSA by analyzing the number of peptides and mis-cleaved peptides together with sequence coverage from nLC-ESI-MS/MS analysis (without connecting the lectin-TPPMR module shown) by varying the amount of BSA injected into the TPPMR at different temperature conditions (25, 37, and 45 °C). BSA was injected from the autosampler at a rate of 3 μL/min with 10 mM ammonium bicarbonate for 30 min. Table 1 shows that the sequence coverage of BSA was larger than 90% at 37 °C with injected amounts of BSA larger than 500 ng, which was the best result among the three temperature conditions. The number of identified peptides (83 from 500 ng BSA) was found to be much larger at 37 °C, compared to 41 at 25 °C and 59 at 45 °C. The list of identified BSA peptides at different temperatures is given in Table S1 and those obtained after different time durations is presented in Table S2. These results show that at least 30 min were needed to achieve good efficiency of online digestion. The digestion efficiency with TPPM was much more enhanced than that with the PS-MAn copolymer alone or in-solution digestion method (sequence coverage and number of identified peptides of BSA are given in Table S3 and comparison of all methods is given in Table S4), and even more improved than that achieved with the PS-MAn membrane described in the previous study.[29] This improvement in efficiency possibly arose from the increased confinement effect upon adopting thermoresponsive PS-MAn-PNIPAm copolymers and the relatively large number of mis-cleaved peptides were likely due to the reduced reaction times. Figure S6a illustrates the proposed mechanism of the restructuring of the TPPM upon increasing the temperature. When the temperature was lower than the lower critical solution temperature (25 °C) of PNIPAm, the PNIPAm moiety of the copolymer stretched into the aqueous solution. However, the PNIPAm moiety curled up to form micelle cavities at a higher temperature (37 °C). After the enzyme was immobilized onto the membrane, the PNIPAm-based micelle cavities exhibited a confinement effect and enhanced the interactions between the enzyme and the substrate. Therefore, the protein digestion efficiency with TPPM was significantly improved in comparison to the PS-MAn membrane.

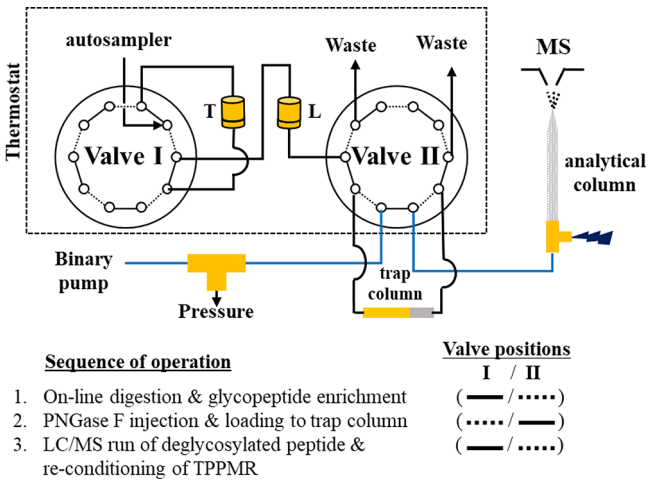

Figure 1. Configuration of the serial TPPMRs for online proteolysis and glycopeptide enrichment prior to nLC-ESI-MS/MS, T: Trypsin immobilized TPPMR. L: Lectin immobilized TPPMR (see detailed structures of TPPMR in Figure S8).

Efficiency of Glycopeptide Enrichment by Lectin-Immobilized TPPMR. The performance of lectin-immobilized TPPMR for glycopeptide enrichment was evaluated by an off-line collection of glycopeptides followed by nLC-ESI-MS/MS analysis. At first, 90 μL of the 5 μg AGP peptide mixtures prepared from in-solution protein digestion were loaded into the lectin-TPPMR for lectin-glycopeptide complexation. The loading flow rates were varied as 6.0, 3.0, and 1.5 μL/min and a binding buffer solution (10 mM ammonium bicarbonate (ABC) with DTT and metal ions (Ca^{2+}, Mn^{2+}, and Mg^{2+}) at pH 7.4 was utilized. Glycopeptides that were not specifically captured by the lectin mixtures and the nonglycosylated peptides that passed during loading were collected (hereafter referred to as the breakthrough fraction) and deglycosylated by mixing with 3 μL (10 unit/μL) of PNGase F at 37 °C for 2 h. After deglycosylation, PNGase F was removed and the entire amount was analyzed by nLC-ESI-MS/MS. For collection of the glycopeptides bound to lectin-TPPMR, the same volume of PNGase F was delivered to the TPPMR at 10 μL/min for 1 min and then maintained at 0.05 μL/min for 30 min to ensure a complete deglycosylation reaction. The injected PNGaseF was detected in a small amount in the online deglycosylation run (Figure S9a and d), but most of them were detected in the fraction collected during the reconditioning process in the first 15 min (Figure S9b and e). After 15 min, no additional PNGase F was detected (Figure S9c and f). This suggested that a small amount of PNGase F was trapped during online deglycosylation, but it was mainly removed during reconditioning. Next, the deglycosylated peptides were collected for 5 min by delivering 10 mM ABC solution without DTT and metal ions at pH 8.4 and 5 μL/min (enrichment fraction). The nLC-ESI-MS/MS analysis of the breakthrough fraction collected at 6.0 μL/min in Figure 2a showed the detection of three glycopeptides (peaks 1, 4, and 5) in addition to two nonglycopeptides (peaks 2 and 3), which indicated that the AGP glycopeptides were not captured efficiently by TPPMR. However, the three glycopeptides were not detected from the fractions of lower flow rates (Figure 2b and Figure 2c) and therefore, the rate of 3 μL/min for 30 min was utilized for protein loading and glycopeptide enrichments.

The effect of temperature on the enrichment of glycopeptides was evaluated at 25, 37, and 45 °C with the loading flow rate of 3 μL/min. From the enrichment fraction, none of the five glycopeptides were detected at 25 °C, but four of the *N*-glycopeptides (56, 72, 93, 103N) were detected at 37 °C. Among them, the extracted ion chromatograms (EICs) of the two glycopeptides, namely, QDQcIYn*TTYLNVQR (95N, m/z = 959.6) and SVQEIQATFFYFTPn*K (72N, m/z = 961.5), were obtained at different temperatures (Figure 3). It was found that the enrichment of glycopeptides was enhanced at 37 °C. This was a result of the confinement effect of the PNIPAm moiety, which formed micelle cavities of TPPM. However, further increase in the temperature to 45 °C could induce a collapse of the micelle cavities, which would hinder the glycopeptide complexation or deglycosylation reaction by PNGase F. This scenario was evidenced by the increase in the contact angle (as shown in Figure S7) from 64.0 ± 7.2° at 25 °C to 92.2 ± 4.4° at 45 °C, which indicated that the PNIPAm moiety was structured such that it formed hydrophobic micelle cavities. Because of the lower critical solution temperature of PNIPAm, the best glycopeptides detection could be realized at 37 °C. The low efficiency of the system at 25 °C may also arise from the decreased activity of PNGase F at a lower temperature. The peptides and glycopeptides identified at different temperature

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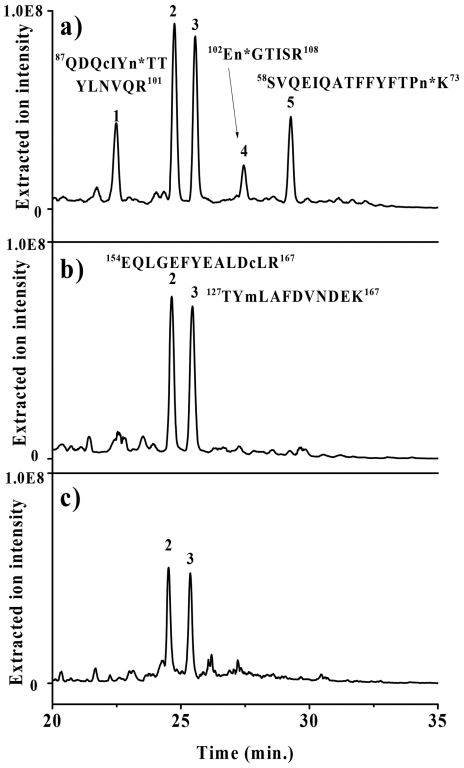

Figure 2. EICs of five different AGP peptides in the breakthrough fraction obtained by varying the loading flow rates (fixed volume) as (a) 6 μL/min for 15 min, (b) 3 μL/min for 30 min, and (c) 1.5 μL/min for 60 min. Peaks 1, 4, and 5 represent the *N*-linked glycopeptides of AGP containing 95N, 103N, and 72N sites, respectively. Peaks 2 and 3 are those of nonglycopeptides.

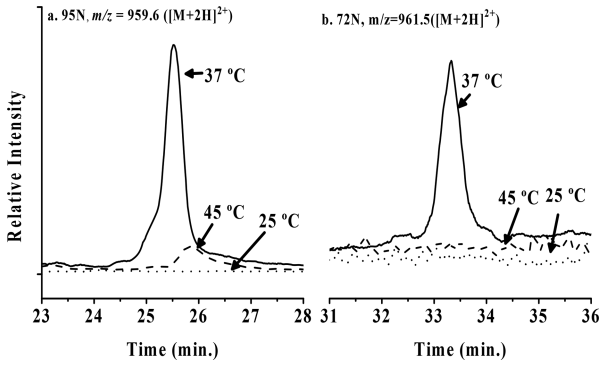

Figure 3. Effect of temperature on the sequential enrichment of AGP glycopeptides followed by deglycosylation with PNGase F, represented by the superimposed EICs of (a) QDQcIYn*TTYLNVQR (95N, m/z = 959.6) and (b) SVQEIQATFFYFTPn*K (72N, m/z = 961.5).

conditions are listed in Table 2 and the average recovery of the four *N*-linked glycopeptides of AGP was 83.2 ± 6.0%, which is given in Table S5.

Online Serial TPPMRs-nLC-ESI-MS/MS for Protein Digestion and Glycopeptide Enrichment. Online protein digestion and simultaneous enrichment of glycopeptides, followed by deglycosylation were carried out with AGP at 37 °C. In this process, AGP was first denatured by dissolving it in the binding buffer for 30 min. Next, 5 μg of the denatured AGP was loaded onto the trypsin-TPPMR from the autosampler at a rate of 3 μL/min for 30 min using the same buffer solution with the valve positions (I: solid and II: dotted lines) in Figure 1. The digested peptides eluted from trypsin-TPPMR were continuously delivered to lectin-TPPMR. At this stage, the unbound glycopeptides along with nonglycosylated peptides passed through the lectin-TPPMR and into the waste. Thus, these breakthrough species were collected and treated with PNGase F to investigate the degree of passage of glycopeptides by nLC-ESI-MS/MS. After the enrichment of glycopeptides, the valve positions were changed (I, dotted; II, solid) for the delivery of PNGase F (3 μL, 10 unit/μL) from the autosampler to the lectin-TPPMR for deglycosylation by using 10 mM ABC buffer. PNGase F was allowed to stay in the lectin-TPPMR for 30 min by delivering the buffer solution at 0.05 μL/min. Then, the pump flow rate was increased to 5 μL/min for 5 min to transfer the deglycosylated peptides to the C18 trap column. Finally, the valve position was changed to (I: solid and II: dotted lines) and the gradient elution of nLC-ESI-MS/MS started. During separation, both TPPMRs were reconditioned by sequential washing with two ABC buffer solutions (pH 5.5 and 8.4) at 10 μL/min for 10 min each. This washing process was repeated thrice and then the binding buffer solution was pumped to the TPPMRs for 20 min. The role of the different pH buffer solutions containing metal ions was to resume the binding capacity of lectin with glycans.[35,36]

Figures 4a–c illustrate the comparison between the base peak chromatograms obtained from the breakthrough fraction and the online deglycosylated peptides along with the corresponding MS spectra obtained during t_r = 22.0–28.0 min. It can be seen in Figure 4c that the three *N*-linked glycopeptides (72N, 93N, and 103N) of AGP were detected by online deglycosylation analysis while the breakthrough fraction contained most of nonglycosylated peptides, all of which were identified from MS/MS experiments. Among the three glycopeptides, when the extracted ion chromatograms (EICs) of QDQCIYn*TTYLNVQR (m/z = 930.5, 93N) obtained from both runs were superimposed in Figure 4d, it was evident that this glycopeptide was not detected in the breakthrough fraction. The data dependent CID spectrum of the 93N glycopeptide at t_r = 22.8 min is shown in Figure 4e. Furthermore, the other two glycopeptides were also not found in the breakthrough fraction. This confirmed that the online enrichment and deglycosylation of glycopeptides were selectively achieved in conjunction with the online proteolysis by using two serial TPPMRs.

Possible carry-over of the remaining glycopeptides in TPPMR was examined by collecting the fractions during the reconditioning (washing) step after each of three repeated deglycosylation runs. Figure 5 compares the EICs of QDQCYn*TTYLNVQR obtained from online deglycosylation run, the breakthrough fraction, and the collected fraction during the washing step in the three consecutive runs of 5 μg AGP. The target glycopeptides were not detected at all in the breakthrough and washing fractions, as shown in Figure 5. Online proteolysis and deglycosylation experiments were further repeated, but the same glycopeptide started to appear in the breakthrough fraction at a very low level (Figure S10), showing the reduced binding affinity for glycopeptides in the fourth and fifth runs. The variation in the peak areas of the three repeated runs of Figure 5a was 3.1%, which implied that the online proteolysis and deglycosylation method was reproducible.

Finally, the online serial TPPMRs-nLC-ESI-MS/MS method was applied to a human plasma proteome sample, from which albumin and IgG had been depleted. In this test, 3 μL of the depleted plasma sample, which was equivalent to 1.5 μL of the original plasma sample because of dilution, was loaded on the

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Table 2. Comparison of the AGP Peptides Identified in the Breakthrough Fraction (BF) and Enrichment Fraction (EF) on Lectin-TPPMR, in Comparison to the Results without Using TPPMR by nLC-ESI-MS/MS Analysis

		25 °C		37 °C		45 °C	
AGP peptides	W/O TPPMR	BF	EF	BF	EF	BF	EF
^{181}DKcEPLEKQHEK^{192}	O	O	O				
^{181}DKcEPLEK^{188}	O	O		O			
^{179}KDKcEPLEK^{188}		O				O	O
^{109}YVGGQEHFAHLLILR^{123}	O	O		O			
^{195}KQEEGES^{201}	O	O		O			
^{171}SDVVYTDWKK^{180}	O	O		O			
^{171}SDVVYTDWK^{179}	O	O					
^{139}NWGLSVYADKPETTK^{153}	O	O		O			
^{154}EQLGEFYEALDcLR^{167}	O	O			O		
^{127}TYMLAFDVNDEK^{138}	O	O		O		O	
^{126}DTKTYmLAFDVnDEK^{138}		O					
^{43}WFYIASAFR^{51}	O		O	O	O	O	O
^{74}TEDTIFLR^{81}				O		O	
^{74}TEDTIFLREYQTR^{86}	O	O		O		O	
^{189}QHEKER^{194}	O	O		O			O
^{183}cEPLEK^{188}	O	O		O		O	
^{52}NEEYn*K^{57} (56N)	O				O	O	O
^{58}SVQEIQATFFYFTPn*K^{73} (72N)	O	O		O	O		
^{87}QDQcIYnTTYLn*VQR^{101} (93N)	O	O			O	O	O
^{102}En*GTISR^{108} (103N)	O				O		
number of nonglycopeptides/glycopeptides	13/4	14/2	2/0	11/1	2/4	7/1	3/2

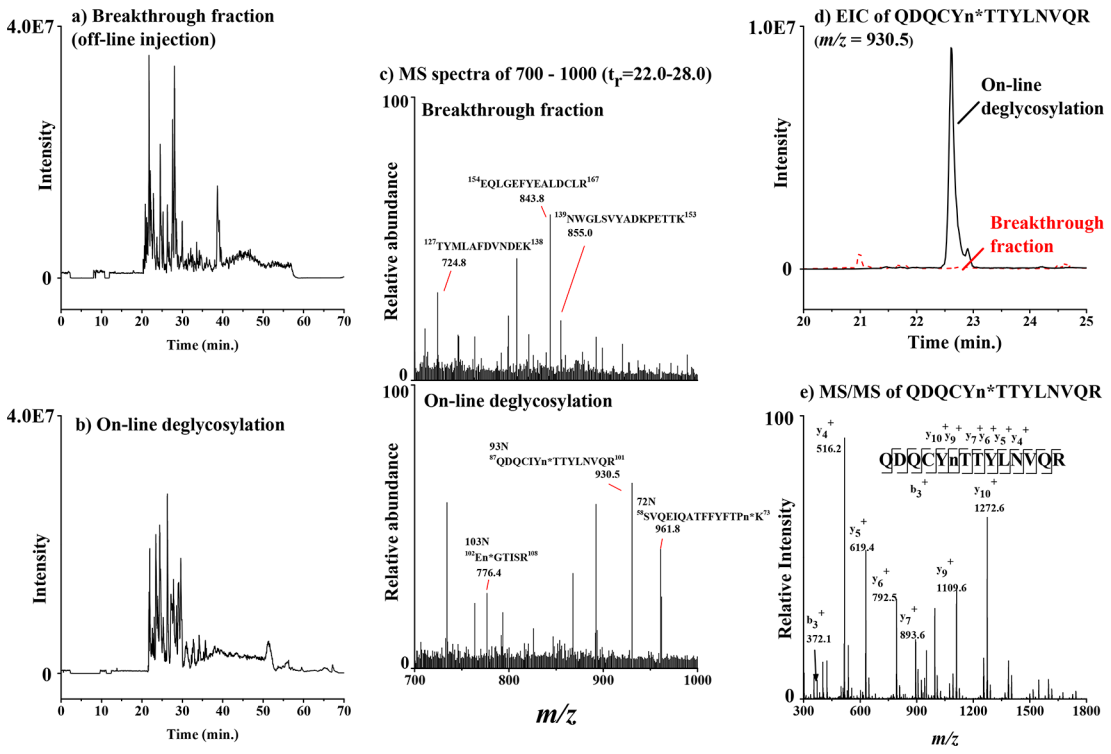

Figure 4. Base peak chromatograms of AGP peptides from the (a) breakthrough fraction (off-line analysis), (b) online deglycosylation of serial TPPMR-nLC-ESI-MS/MS, (c) MS spectra of m/z 700−1000 at t_r = 22.0−28.0. (d) EIC of QDQCYn*TTYLNVQR (m/z = 930.5) compared between the breakthrough fraction (red line) and online deglycosylation runs (black line), and (e) data-dependent CID spectra of the deglycosylated peptide ion (m/z = 930.5) at t_r = 22.8 min identified as QDQCYn*TTYLNVQR.

online system for proteolysis and glycopeptide enrichment. The analysis was repeated three times. For comparison with off-line enrichment, a same volume of the sample was in-solution digested, enriched with lectins, and deglycosylated with PNGase F, followed by nLC-ESI-MS/MS analysis. The off-line enrichment was repeated thrice. Online serial TPPMRs-nLC-ESI-MS/MS analysis of a depleted human plasma sample resulted in the identification of 262 *N*-glycopeptides out of the total 605

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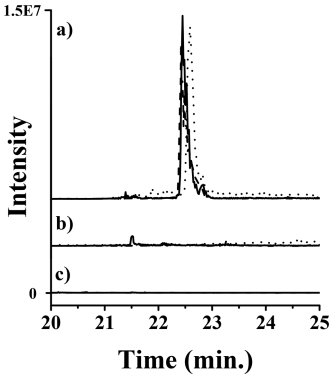

Figure 5. EICs of QDQCYn*TTYLNVQR (m/z = 930.5) obtained from three consecutive runs of 5 μg AGP from the (a) online deglycosylation, (b) breakthrough fraction, and (c) fraction collected during reconditioning of TPPMR.

peptides (43.3%) and 155 glycoproteins from total 277 proteins (Table S6), which was a much larger number than the 115/84 glycopeptides/glycoproteins determined from in-solution enrichment for comparison (BPCs in Figure S11). It has been reported that the enrichment of glycopeptides utilizing solid phase enrichment materials resulted in identification of 194/104 glycopeptides/glycoproteins with the use of hydrophilic chitosan microspheres[37] and 424/140 with hydrophilic mesoporous silica materials,[38] both from 2 μL of the human serum. While the number of identified glycopeptides from online enrichment was somewhat lower than the reported results,[39−41] the online method resulted in the identification of more glycoproteins. Moreover, the present method provided a fully automated method from proteolysis to glycopeptide enrichment and subsequent deglycosylation with only 1.5 μL of human plasma.

CONCLUSIONS

The present work demonstrated a fully automated analytical protocol for the online proteolysis of proteins followed by the sequential enrichment of glycopeptides prior to nLC-ESI-MS/MS analysis. This method was then utilized for the glycoproteomic analysis of human plasma or serum. The use of two serial TPPMRs was based on the immobilization of trypsin and lectins on the TPPM in each enzyme reactor, enabling protein digestion and subsequent enrichment of glycopeptides in less than an hour, which was much less than the time required for a series of digestion and enrichment processes in solution. By incorporating the thermoresponsive PS-MAn-NIPAM copolymer as the membrane substrate on the nylon surface, micelle cavities formed by the PNIPAm moiety could be manipulated at elevated temperatures, rendering higher performances in accommodating the enzyme and lectins at 37 °C as compared to using PS-MAn alone. An initial evaluation of the TPPMR implementing a lectin mixture (ConA/WGA) immobilized membrane with AGP showed that the enrichment of *N*-glycopeptides and deglycosylation with PNGase F were best performed at 37 °C. The application of the online serial TPPMRs-nLC-ESI-MS/MS analysis resulted in an identification of a total of 262 glycopeptides out of 155 glycoproteins from 1.5 μL of the human plasma sample. The present TPPMR can be developed further into a versatile membrane reactor, in which the membranes immobilized with lectins or antibodies can be replaced in a simple step once the immobilization of bioactive species and storage method is well optimized. Especially, the developed technique can be effectively applied for the characterization of glycopeptides from a limited volume of a fresh biological sample such as mouse serum or urinary proteome, and for the development of cancer specific biomarker candidates of glycoproteins once a relative quantitation method is integrated.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.7b04273.

Synthesis of PS-MAn and PS-MAn-NIPAm, in-solution digestion and glycopeptide enrichment of plasma sample, nLC-ESI-MS/MS, RAFT synthesis and ^{1}H NMR and FT-IR spectra of PS-MAn and PS-MAn-NIPAm, fabrication of TPPM by breath figure method and SEM image of TPPM, effects of temperature, immobilization time, layers of TPPM, enzyme concentration, the incorporation of PNIPAM, and ratio of PNIPAm/PS-MAn on the hydrolysis efficiency of enzyme immobilized TPPM, schematic illustration of the thermosensitive properties of TPPM, property of TPPM evaluated by contact angle, TPPMR module assembled with two layers of Nylon sheet coated with PS-MAn-NIPAm copolymer only for enzyme immobilization, the inner structure of the trypsin−TPPMR, the inner structure of the lectin−TPPMR, base peak chromatograms of nLC run, EICs of QDQCYn*TTYLNVQR, full MS spectrum at each run, base peak chromatograms of tryptic glycopeptides, amino acid sequences of BSA, BSA digestion efficiency, sequence coverage, the number of identified peptides, and the number of mis-cleaved peptides, recovery of four AGP peptides in deglycosylation step, and identified glycopeptides and glycoproteins from depleted pooled plasma sample (PDF)

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Notes

The authors declare no competing financial interest.

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