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RESEARCH PAPER

Highly efficient N-glycoproteomic sample preparation by combining C_{18} and graphitized carbon adsorbents

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Abstract Conventional N-glycoproteome analysis usually applies C_{18} reversed-phase (RP) adsorbent for sample purification, which will lead to unavoidable sample loss due to the high hydrophilicity of N-glycopeptides. In this study, a porous graphitized carbon (PGC) absorbent was combined with a C_{18} adsorbent for N-glycopeptide purification in comprehensive N-glycoproteome analysis based on the hydrophobic and polar interactions between carbon and N-glycans. It was observed that the small hydrophilic N-glycopeptides that cannot retain onto C_{18} adsorbent can be captured by the graphitized carbon, while the large hydrophobic N-glycopeptides that cannot retain onto the graphitized carbon can be feasibly captured by the C_{18} adsorbent. Comparing with sample purification by using C_{18} adsorbent only, 28.5 % more N-glycopeptides were identified by combining both C_{18} and PGC adsorbents. The C_{18}-PGC strategy was further applied for both sample purification and pre-fractionation of a complex protein sample from HeLa cell. After hydrophilic interaction chromatography enrichment, 1,484 unique N-glycopeptides with 1,759 unique N-glycosylation sites were finally identified.

Keywords Porous graphitized carbon · C_{18} adsorbent · N-glycoproteomic sample preparation · Proteomics · LC-MS/MS

Abbreviations

ACN	Acetonitrile
CID	Collision-induced dissociation
Click maltose-HILIC	Click maltose Hydrophilic interaction chromatography
DTT	Dithiothreitol
FA	Formic acid
HILIC	Hydrophilic interaction chromatography
HRP	Horseradish peroxidase
IAA	Iodoacetamide
LC-MS	Liquid chromatography coupled with mass spectrometry
LTQ	Linear ion trap
MALDI-TOF MS	Matrix-assisted laser desorption ionization-time-of-flight mass spectrometry
PGC	Porous graphitized carbon
PNGase F	Peptide N-glycosidase F
RP	Reversed phase
SPE	Solid-phase extraction
TFA	Trifluoroacetic acid

Instruction

Protein glycosylation is one of the most important posttranslational modifications (PTMs) and plays crucial roles in different biological processes [1, 2], and the aberrant changes in protein glycosylation in human are always associated with

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diseases, such as cancer [3, 4]. Therefore, different technologies of glycoproteome analysis are rapidly developed for pathological process investigation and disease biomarker discovery in recent years [5]. Sample preparation, including protein extraction, digestion, purification, pre-fractionation, and specific enrichment, is usually the first step of glycoproteome analysis and is also crucial to the final performance of the whole analysis.

Bottom-up strategy is the most usually utilized in current proteome analysis, and the protein samples are digested to peptide mixture before liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) detection. As the protein digest solution always contains a high concentration of salt and other types of contaminants (for protein extraction and pH buffering), sample purification is unavoidable before LC separation. Conventionally, different types of solid-phase extraction (SPE) technologies by using C_{18} reversed-phase (RP) adsorbent are utilized for protein sample purification, and most of the peptides or proteins can be purified by C_{18} adsorbents with good recovery [6, 7]. However, the sample loss during C_{18} SPE purification will significantly increase when some extremely hydrophilic samples are purified, such as the N-glycopeptides with hydrophilic glycan chains [8, 9]. Although hydrophilic interaction liquid chromatography (HILIC) is widely applied to enrich or separate hydrophilic analytes such as N-glycopeptides [10], a high concentration of salt and contaminants will greatly decrease the retention of analytes onto HILIC adsorbents. Therefore, it is important to find good alternative SPE strategies for a highly efficient purification of hydrophilic N-glycopeptides. PGC is a conducting crystalline material which exhibits effective retention capability for polar compounds [9]. Recently, the porous graphitic carbon (PGC) adsorbents have shown excellent ability for retaining extremely hydrophilic compounds, such as sugars, glycans, and hydrophilic peptides, due to the special polar interaction between carbon and glycopeptides [11, 12]. The PGC adsorbents have already been utilized for glycan [13, 14] and glycopeptide [9, 8, 15] separation. Lewandrowski et al. reported on a dual gradient system by combining RP and PGC retention modes within a single nano-high-performance liquid chromatography (HPLC) system, and a potential increase in sequence coverage for ribonuclease B was observed due to the additional PGC retention mode [8]. However, only standard glycoprotein samples were analyzed by using PGC adsorbents until now [14, 16], and the performance of a PGC adsorbent in comprehensive N-glycoproteome analysis of real complex protein samples still needs investigation.

Herein, the PGC adsorbent was applied for purification of a complex protein sample from a cell line before N-glycopeptide enrichment and following LC-MS/MS analysis. Comparing with the conventional C_{18} adsorbent, small N-glycopeptides with high hydrophilicity can be better extracted by the PGC adsorbent. However, large N-glycopeptides with high hydrophobicity cannot be captured by the PGC adsorbent, and combining both C_{18} RP and PGC adsorbents in series gave the highest coverage in N-glycoproteome analysis, in which 28.5 % more N-glycopeptides were identified compared with that in the traditional C_{18} SPE strategy. Furthermore, a simple sample pre-fractionation strategy was integrated into the C_{18}-PGC SPE strategy by using a stepwise acetonitrile (ACN) elution to further increase the N-glycoproteome coverage.

Experimental

Chemicals and materials

Horseradish peroxidase (HRP), trypsin (TPCK treated), iodoacetamide (IAA), dithiothreitol (DTT), trifluoroacetic acid (TFA), ammonium bicarbonate (NH_4HCO_3), formaldehyde, and sodium cyanoborohydride were purchased from Sigma (St. Louis, MO). Ethylenediaminetetraacetic acid (EDTA), ethylene glycol bis(2-aminoethyl) tetraacetic acid (EGTA), and phenylmethanesulfonyl fluoride (PMSF) were purchased from Amresco (Solon, OH, USA). PNGase F was obtained from New England Biolabs (Ipswich, MA), and formic acid (FA) was from Fluka (Buches, Germany). 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). C_{18} AQ beads (3, 5, and 40 μm, 120 Å) were purchased from Daiso (Osaka, Japan). GELoader tips (20 μL) were purchased from Eppendorf (Hamburg, Germany). PGC beads obtained from HyperSep Hypercarb (PGC) SPE columns were purchased from Thermo Fisher Scientific (San Jose, CA). All the water used in experiments was purified with a Milli-Q system from Millipore (Milford, MA).

Preparation of C_{18} RP, PGC, and C_{18} RP-PGC SPE column

For standard glycoproteins, 20 mg of C_{18} beads (40 μm, 120 Å) or 20 mg of PGC beads (30 μm, 150 Å) were packed into a 1-mL syringe, respectively. The C_{18} RP-PGC SPE column was made by packing 10 mg of C_{18} beads on the top of a 10-mg well-packed PGC column. More adsorbents (at a ratio of 60:1 with protein) were packed for complex samples.

Preparation of HILIC-SPE tips

HILIC-SPE tips were prepared as previously described [17]. Briefly, the GELoader tip was first packed with a small piece of cotton wool as the sieve. Then, 3–5-mg click maltose-HILIC beads (4 μm, 100 Å) were packed into the tip by centrifugation at 4,000g for several minutes. Finally, the tip

was washed by 50 μL of H_2O and equilibrated with 50 μL of 80 % ACN before use.

Comparison of the retention performance of different SPE columns using standard glycoproteins

The standard glycoprotein HRP was firstly denatured by 8 M urea/100 mM TEAB (pH 8.0) and heated in a boiling water bath for 5 min, then diluted them with 100 mM TEAB buffer (pH 8.0), and digested with trypsin as previously described [17, 18].

Fifty micrograms of HRP tryptic digests was loaded onto the C_{18}, PGC, and C_{18} RP-PGC SPE columns, respectively. After washing the column with 600 μL of 100 mM TEAB buffer (pH 8.0), a 1-mL isotope labeling reagent containing 0.04 % formaldehyde, 6 mM cyanoborohydride, and 100 mM TEAB (pH 8.0) was loaded to the SPE column as previously described [19], while light labeling (L) for PGC, middle labeling (M) for C_{18} RP-PGC, and heavy labeling for C_{18} (H). After washing the column with 1 mL of 0.1 % TFA, the peptides were eluted with 600 μL of 0.1 % TFA/80 % ACN. The labeled peptides were mixed together and lyophilized and then redissolved by a loading buffer (1 % TFA/80 % ACN). Sixty microliters of solution equivalent to 30 μg of protein was pipetted into a 3-mg HILIC tip. After centrifuging at 4,000*g* for about 15 min, the HILIC tip was washed with 80 μL of loading buffer and then eluted with 30 μL of 0.1 % TFA. The eluted intact glycopeptides were detected by matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF MS) directly.

HeLa cell protein preparation and glycopeptide enrichment

The HeLa cell proteins were prepared as previously described [18]. For the comparison of glycopeptide extraction performance of different SPE columns, the same amount of samples was cleaned up with the C_{18}, PGC, and C_{18}-PGC SPE column, respectively, eluted with 0.1 % TFA/80 % ACN and lyophilized. For the fractionation, the samples were cleaned up with C_{18}-PGC SPE columns and collected as six fractions, and then lyophilized: 20, 25, 30, 35, 40, and 80 % ACN. The lyophilized HeLa digest was redissolved in a loading buffer (1 % TFA/80 % ACN), and 100 μL of solution equivalent to 50 μg of protein was pipetted into a 5-mg HILIC tip. After centrifuging at 4,000*g* for about 30 min, the HILIC tip was washed with 250 μL of the loading buffer and then eluted with 100 μL of 100 mM NH_4HCO_3 containing 50 U of PNGase F to a microtube by centrifuging at 1,000*g* for 30 min, and incubated overnight. The eluted peptides were finally analyzed by LC-MS/MS.

Mass spectrometry analysis

HRP digest samples were analyzed by AB Sciex 5800 MALDI-TOF/TOF mass spectrometer (AB Sciex, CA, USA) equipped with a pulsed Nd:YAG laser at 355 nm in reflect positive-ion mode; 0.5 μL 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.

Normal HeLa cell samples were analyzed by LTQ Orbitrap Velos (Thermo, San Jose, CA) with Accela 600 HPLC system (Thermo, San Jose, CA). The deglycosylated peptides were loaded on a C_{18} capillary trap column (200 μm i.d.×4 cm) packed with C_{18} AQ beads (5 μm, 120 Å) by a void capillary as previously described [20]. 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 nanoflow LC-MS/MS analysis. The gradient from 5 to 35 % (*v*/*v*) ACN was performed in 90 min. The MS and MS/MS spectra were collected by collision-induced dissociation (CID) at 35 % of energy in a data-dependent mode with one MS scan followed by 20 MS/MS scans.

The fractionated HeLa cell samples were analyzed by Triple-TOF 5600 system (AB Sciex, CA, USA) equipped with a NanoACQUITY UPLC (Waters, USA) for separation, including a 3-cm C_{18} capillary trap column (200 μm i.d.), a 20-cm C_{18} capillary analysis column (75 μm i.d.), and a PicoTip Emitter (New Objective, USA). The flow rate was set at 0.35 μL/min, and the RP gradient was as follows: from 0 to 5 % buffer B (98 % ACN/2 % H_2O/0.1 % FA) for 5 min, from 5 to 25 % buffer B for 80 min, and from 35 to 70 % buffer B for 15 min. After flushing with 70 % of buffer B for 10 min, the separation system was equilibrated by buffer A (98 % H_2O/2 % ACN/0.1 % FA) for 10 min. The instrument was operated in information-dependent acquisition (IDA) mode, with the top 40 precursors (charge state +2 to +5, >50 counts) in each full MS scan (800 ms, scan range 350–1,250 *m*/*z*) subjected to MS/MS analysis (minimum accumulation time 100 ms, scan range 100–1,500 *m*/*z*). Ion exclusion time was set to 30 s.

Database searching

The RAW files collected by Xcalibur 2.1 were converted to *.MGF by Proteome Discoverer (v1.2.0.208, Thermo, San Jose, CA) and searched with Mascot (version 2.3.0, Matrix Science, London, UK). The WIFF files collected by Analyst 1.5.1 were searched with ProteinPilot version 4.1.46 (AB SCIEX, USA). The IPI human 3.80 database was used for both searching. For Mascot, cysteine carboxamidomethylation was set as a static modification of 57.0215 Da; methionine oxidation and asparagine deamination were set as a variable modification 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. For ProteinPilot searching, no variable modification and mass tolerance were set, as the search engine includes all the variable modifications such as Asn and Gln deamidation. The false discovery rate (FDR) for peptide identifications of both searches was controlled to <1 %, and only glycopeptides with N-X-S/T (where X can be any amino acid except Prolin) or rarely the N-X-C motif were considered as highly reliable results and used for investigation.

Results and discussion

The extraction performance of C_{18} and PGC adsorbents for N-glycopeptides

Firstly, the extraction performance of C_{18} and PGC RP adsorbents for N-glycopeptides was investigated by using the HRP tryptic digests. For direct comparison of different SPE purification strategies, triple isotope dimethyl labeling was utilized in our investigation as light labeling for PGC, middle labeling for C_{18}-PGC, and heavy labeling for C_{18} SPE cleaned samples, respectively.

HRP is a glycoprotein with eight N-glycosylation sites, and 70 % of the attached glycans are $GlcNac_2(Fuc)Man_3(Xyl)$ [21]. Equal amounts of HRP digests were purified by the three different SPE strategies and labeled with mass-differentiated dimethyl group. Then, the three different protein samples were mixed together, and the N-glycopeptides were enriched by the HILIC tip as described in the "Experimental" section before MALDI-TOF MS analysis. Finally, it was observed that the extraction performances of the three SPE strategies varied significantly for different N-glycopeptides even if identical N-glycan was attached (Fig. 1). Briefly, for the short N-glycopeptide NVGLNR (the average GRAVY (Grand average of hydropathicity) [22] of this peptide backbone is −0.65) with $GlcNAc_2(Fuc)Man_3(Xyl)$ glycan, only light and middle isotopic forms were observed (Fig. 1-A), which indicated that this N-glycopeptide was too hydrophilic to be retained on the C_{18} RP absorbent. Similar peak intensity of the three isotopic forms was also observed for N-glycopeptides with moderate hydrophobic peptide backbone MGNITPLTGTQGQIR (the average GRAVY was −0.35) and identical N-glycan as described above (Fig. 1-B). However, a further increase of the sequence length and hydrophobicity of peptide backbones significantly decreased the peak intensity of light isotopic form (Fig. 1-C, -D). For the N-glycopeptides GLIQSDQELFSSPNATDTIPLVR and LHFHDCFVNGCDASILLDNTTSFR (the average GRAVY of these two peptide backbones were −0.161 and 0.146) with identical N-glycan, the intensity of light isotopic peaks was much lower than the other two peaks and even missed. This is consistent with that in previous reports that PGC adsorbent cannot capture large hydrophobic glycopeptides [9]. Therefore, the PGC adsorbent exhibits much better extraction efficiency than the C_{18} adsorbent for the small hydrophilic N-glycopeptides, but much worse extraction efficiency for the large

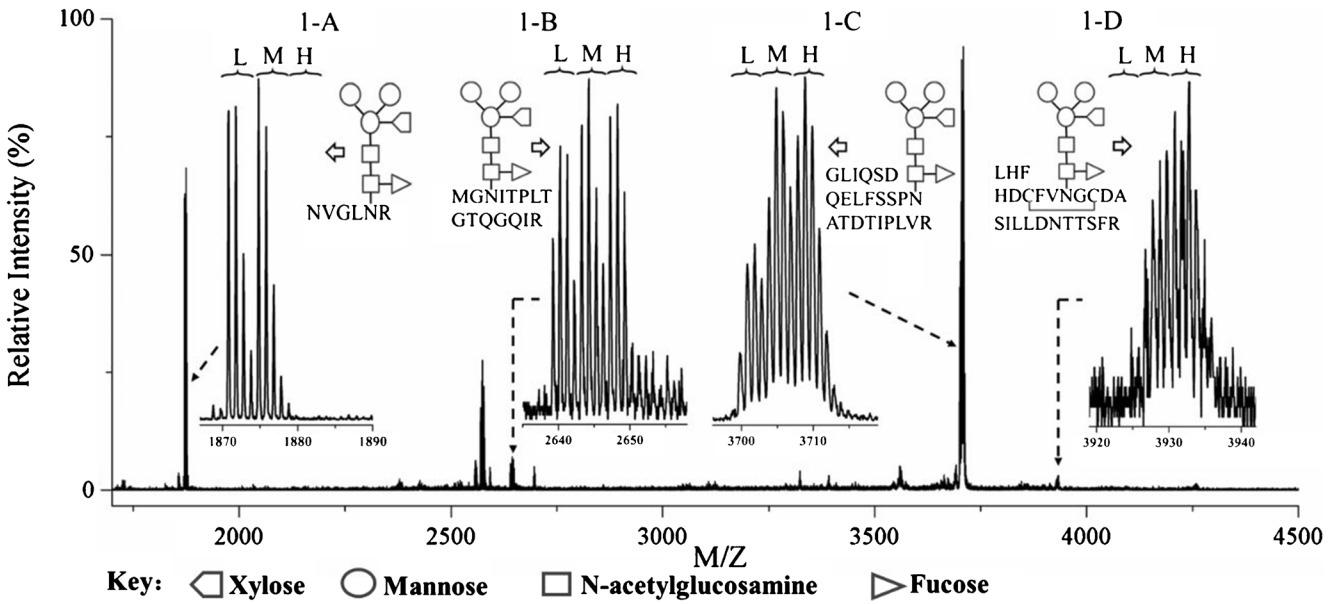

Fig. 1 MALDI-TOF mass spectra for triple labeled glycopeptides of HRP digest. *Inset*: MALDI-TOF mass spectra for glycopeptide NVGLNR-$GlcNAc_2(Fuc)Man_3(Xyl)$ (*1-A*), MGNITPLTGTQGQIR-$GlcNAc_2(Fuc)Man_3(Xyl)$ (*1-B*), GLIQSDQELFSSPNATDTIPLVR-$GlcNAc_2(Fuc)Man_3(Xyl)$ (*1-C*), and glycopeptide LHFHDCFVNGCDASILLDNTTSFR-$GlcNAc_2(Fuc)Man_3(Xyl)$ (*1-D*). Triple isotope dimethyl labeling was utilized as light labeling for PGC SPE (*L*), middle labeling for C_{18}-PGC SPE (*M*), and heavy labeling for C_{18} SPE (*H*) cleaned samples, respectively

Table 1 The numbers of unique N-glycopeptides and N-glycoproteins identified by different SPE strategies

SPE	C_{18}-1	C_{18}-2	PGC-1	PGC-2	C_{18}-PGC-1	C_{18}-PGC-2
Number of unique glycopeptides	284	299	333	331	386	345
Number of glycoproteins	186	191	206	222	242	218
Number of combined glycopeptides	358		401		460	

hydrophobic N-glycopeptides because the interaction between PGC and glycan is not strong enough to well capture the large N-glycopeptides. In addition, the middle isotopic peak always exhibited the highest peak intensity for either small hydrophilic or large hydrophobic N-glycopeptides in our investigation, which indicated that combining both C_{18} and PGC adsorbents in the same SPE column is the best strategy for sample purification of N-glycoproteome analysis.

Comparison of different SPE sample purification strategies in N-glycoproteome analysis

The PGC adsorbents are only applied for N-glycopeptide purification and separation for standard protein samples until now. In this study, tryptic digest of the HeLa cell protein was utilized to investigate the sample purification performance of PGC, C_{18}-PGC, and C_{18} SPE strategies, respectively, and HILIC enrichment was applied after each of these SPE strategies to eliminate the influence of non-glycopeptides. Only N-glycopeptides with N-X-S/T (where X can be any amino acid except Proline) or rarely the N-X-C motif were considered as highly reliable results and used for comparison in all of our investigations.

The numbers of N-glycopeptides and N-glycoproteins that were identified by different SPE strategies were shown in Table 1 (detailed dates were in the Electronic Supplementary Material Table S1 and Fig. S1). Obviously, comparing with sample purification by using the C18 adsorbent only, both PGC and C18-PGC exhibited higher N-glycoproteome coverage, and the numbers of identified N-glycopeptides increased by 12.0 and 28.5 %, respectively. The overlaps of N-glycosylation sites identified by the three different SPE strategies were also investigated (Fig. 2); 245 N-glycosylation sites could be mapped by all of the three SPE strategies, and the C18-PGC SPE strategy covered 79.9 and 82.0 % of N-glycosylation sites identified by C18 and PGC SPE strategy, respectively (Fig. 2 and Electronic Supplementary Material Table S1). Obviously, the C_{18}-PGC SPE strategy also gave the best performance in large-scale N-glycoproteome analysis. In contrast to identical N-glycan structures that are attached on different glycosylation sites within standard glycoprotein HRP, the N-glycan structures exhibit huge diversity within a complex cell line sample. Therefore, there are no clear trends in peptide backbone GRAVY values for different SPE strategies (data not shown).

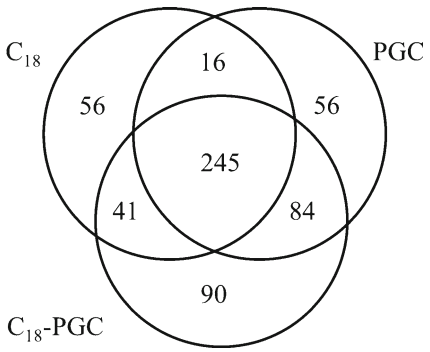

Fig. 2 The overlap of identified N-glycosylation sites by different SPE strategies

Sample purification and pre-fractionation by C_{18}-PGC SPE strategy for N-glycoproteome analysis

Although the MS and HPLC systems are rapidly developed in the past decades, it is still impossible to characterize all the proteins and protein PTMs within the complex biological samples. Therefore, decreasing the sample complexity by pre-fractionation is essential to comprehensive proteome analysis, especially for proteome PTM analysis. The C_{18}-PGC SPE column exhibited the best performance of sample

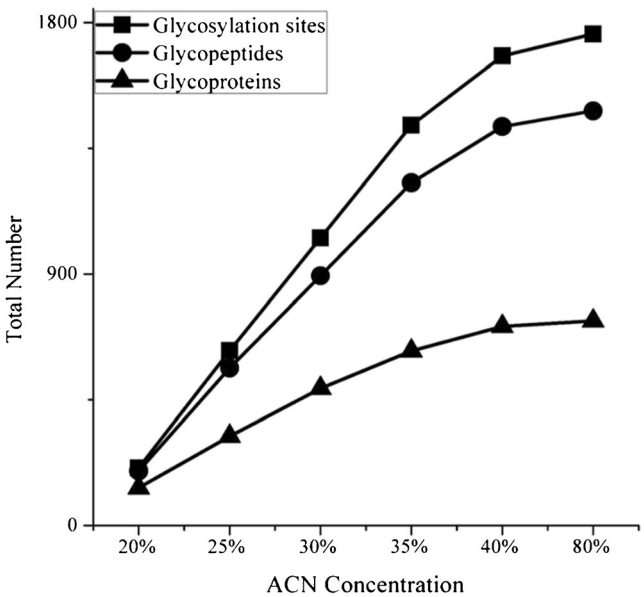

Fig. 3 The total number of identified N-glycosylation sites, N-glycopeptides, and N-glycoproteins along with the increasing ACN concentration steps

purification in the N-glycoproteome analysis as described above. Thus, we further developed a simple sample pre-fractionation strategy just on the same C_{18}-PGC SPE column after sample purification to increase the N-glycoproteome coverage. Briefly, six solutions with different ACN concentrations were applied to stepwisely elute the purified peptides from the C_{18}-PGC SPE column before subsequent HILIC enrichment as described in the "Experimental" section. After nanoflow LC-MS/MS analysis and controlling the FDR <1 % for N-glycopeptides' identification, 1,248 and 1,099 unique N-glycopeptides with 1,377 and 1,191 unique N-glycosylation sites were feasibly identified in two independent analyses of 300-μg HeLa cell protein samples, respectively, and the numbers of corresponding N-glycosylated proteins were 609 and 550, respectively (see the Electronic Supplementary Material Table S2). Finally, 1,484 unique glycopeptides with 1,759 unique glycosylation sites had been obtained in the two replicate analyses (see the Electronic Supplementary Material Table S3). It could be seen that the total number of identified N-glycosylation sites, N-glycopeptides, and N-glycoproteins almost linearly increased with the increasing of ACN steps, which indicated the high efficiency of the pre-fractionation strategy (Fig. 3 and Electronic Supplementary Material Fig. S2). Therefore, a fast sample pre-fractionation strategy was successfully integrated into the C_{18}-PGC SPE strategy to significantly increase the N-glycoproteome coverage, and all the procedures of sample purification and pre-fractionation were performed onto the same C_{18}-PGC SPE column, which greatly simplify the manual operation and decrease the sample loss.

Conclusion

Sample preparation is essential to the performance of proteome analysis, especially for proteome PTM analysis because the PTM sites are always low in abundance. In this study, we demonstrated that the C_{18}-PGC SPE strategy gave the best performance of sample purification in N-glycoproteome analysis of complex cell line sample. Comparing with sample purification by using C_{18} or PGC adsorbent alone, the C_{18}-PGC SPE strategy could identify 28.5 and 14.7 % more N-glycopeptides, respectively. After sample purification with the C_{18}-PGC SPE column, the tryptic peptides could be further pre-fractionated into six fractions with different ACN steps, and finally, 1,484 unique glycopeptides with 1,759 unique glycosylation sites were obtained in two replicate analyses. The good pre-fractionation performance was demonstrated by the linear increase of total identified N-glycosylation sites along with the different ACN steps. Therefore, we believe that the C_{18}-PGC SPE strategy is suitable for N-glycoproteome analysis in both sample purification and pre-fractionation.

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