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Technical Note

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Highly Efficient Release of Glycopeptides from Hydrazide Beads by Hydroxylamine Assisted PNGase F Deglycosylation for N-Glycoproteome Analysis

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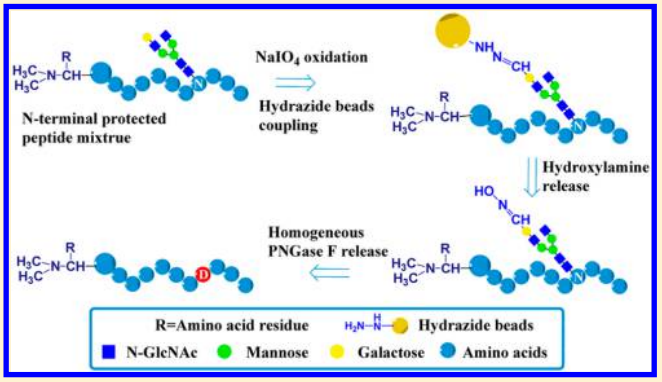
Supporting Information

ABSTRACT: Selective enrichment of glycopeptides from complex sample followed by cleavage of N-glycans by PNGase F to expose an easily detectable mark on the former glycosylation sites has become the popular protocol for comprehensive glycoproteome analysis. On account of the high enrichment specificity, hydrazide chemistry based solid-phase extraction of N-linked glycopeptides technique has sparked numerous interests. However, the enzymatic release of glycopeptides captured by hydrazide beads through direct incubation of the beads with PNGase F is not efficient due to the inherent steric hindrance effect. In this study, we developed a hydroxylamine assisted PNGase F deglycosylation (HAPD) method using the hydroxylamine to release glycopeptides captured on the hydrazide beads through the cleavage of hydrazone bonds by transamination followed with the PNGase F deglycosylation of the released glycopeptides in the free solution. Because of the homogeneous condition for the deglycosylation, the recovery of deglycosylated peptides (deglycopeptides) was improved significantly. It was found that 27% more N-glycosylation sites were identified by the HAPD strategy compared with the conventional method. Moreover, the ratio of identified N-terminal glycosylated peptides was improved over 5-fold.



Protein N-glycosylation is an important post-translational modification which significantly effects on protein folding, structure, stability, and activity.[1,2] It plays protective, structural, and stabilizing roles in the living cells and is related to many biological mechanisms including immune response, cell division, growth, differentiation, and apoptosis.[3−5] It is well acknowledged that abnormal N-glycosylation is closely related to oncogenesis, tumor progression, and other serious disease, and to date, the vast majority of cancer biomarkers used for medical diagnosis are glycoproteins.[6,7] Therefore, comprehensive profiling of N-glycoproteome in the biological samples is of high imperative. While this work still challenge the state of art glycosylation identification technology due to the high dynamic range of protein concentration in biological samples and the complexity and heterogeneity of the protein N-glycosylation.[8,9] To achieve comprehensive N-glycoproteome analysis, selective enrichment of N-glycopeptides from the complex sample followed with the cleavage of N-glycans by PNGase F to expose an easily detectable mark on the former glycosylation site has become the popular protocol for comprehensive N-glycoproteome analysis.[10−12]

To date, there are mainly four glycopeptide enrichment approaches used for the N-glycoproteome analysis: (1) lectin affinity chromatography (LAC);[13] (2) titanium dioxide chromatography (TiO_2);[14,15] (3) hydrophilic interaction chromatography (HILIC);[16−18] and (4) chemical reaction based method using the hydrazide chemistry method.[19−22] It was reported that these methods are highly complementary and can be used together to improve the glycoproteome coverage.[23−25] Among these methods, hydrazide chemistry based solid-phase extraction of the N-linked glycopeptides (SPEG) technique is particularly promising due to its good enrichment specificity, which benefits from the formation of covalent bonds between glycans of glycoproteins/glycopeptides and hydrazide

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groups on the solid support.[26] Since glycoproteins/glycopeptides are covalently bound to hydrazide beads, nonspecific bound proteins or peptides on beads can be well removed by harsh washing procedures. The additional advantage of this SPEG method is that it integrated all sample preparation procedures into the solid support, thus extra steps for desalting and buffer exchanging are avoided and the sample loss and contamination could be greatly reduced.[27] As a result, the high sensitivity can be obtained.

In usual N-glycoproteome analysis, peptide-N-glycosidase F (PNGase F) is used for enzymatic cleavage of the glycosidic bond between N-glycan and its attached peptide to facilitate the identification of the glycopeptide sequence and glycosylation site.[28] The PNGase F deglycosylation step can be classified into two types: one is homogeneous deglycosylation where the glycopeptides are presented in the free solution when PNGase F is added and the other one is heterogeneous deglycosylation where the glycopeptides are captured on beads when deglycosylation is performed. The deglycosylation step in the LAC, TiO_2, and HILIC methods generally belongs to the first type as the glycopeptides are typically eluted from beads to free solution for deglycosylation.[13,15,16] While as for the hydrazide chemistry method, glycopeptides are covalently bound on hydrazide beads, the deglycosylation are always performed in heterogeneous condition.[19,21] Thus, in the conventional hydrazide chemistry method, the PNGase F may have difficulties in accessing the glycosidic bonds due to the steric hindrance and the deglycosylation would not be as efficient as it is performed in the homogeneous condition. In this study, we presented a Hydroxylamine Assisted PNGase F Deglycosylation (HAPD) strategy to enable the enzymatic deglycosylation of glycopeptides performed in homogeneous conditions for the hydrazide chemistry method. Compared with directly releasing deglycopeptides from hydrazide beads by PNGase F, the HAPD strategy allowed the enzyme to freely access glycosidic bonds in solution and thereby significantly improved the deglycosylation efficiency. Applying mouse liver lysate as the sample, we compared the performance of this new HAPD method with that of the conventional hydrazide chemistry method. It was found that 889 glycosylation sites were obtained from the HAPD strategy, while only 700 glycosylation sites were identified in the conventional method. The new HAPD strategy yields 27% more glycosylation site identifications than the conventional method. Moreover the ratio of identified N-terminal glycosylated peptides of the hydrazide chemistry method has also been improved by over 5-fold (from 0.84% to 5.31%).

EXPERIMENTAL SECTION

Glycopeptide Enrichment. To avoid the oxidation of N-terminal serine/threonine peptides, all of the tryptic digests used in this work were subjected to a peptide N-terminal protection approach.[29] Briefly, tryptic peptides of standard glycoprotein or mouse liver extracts were dissolved in triethylammonium bicarbonate (TEAB,100 mM) buffer with a concentration of 1 mg/mL. Each protection approach, 100 μL of formaldehyde (4%, v/v) was added in 1 mg of the tryptic peptides followed with mixing of 100 μL of newly prepared sodium cyanoborohydride (0.6 M). The reaction mixture was shook for 1 h at ambient temperature. Then, the residual labeling reagents were quenched by addition of 20 μL of ammonia (10%, v/v). After the protection approach, the reaction mixture was acidified by formic acid to pH 2−3 and then desalted by the SPE device and dried in a vacuum centrifugal concentration system.

The enrichment of the glycopeptide by the hydrazide chemistry method was performed in accordance with the approach reported by Tian et al.[21,27] Briefly, each aliquot of 50 μg of peptides from mouse liver was redissolved in 100 μL of oxidation buffer (100 mM NaAc, 150 mM NaCl, pH = 5.5) followed by addition of $NaIO_4$ with a final concentration of 2 mM (three technical replicates were conducted for each experiment). The reaction mixture was incubated in the dark for 1 h and the excess $NaIO_4$ was consumed by sodium thiosulfate. Then the reaction mixture was mixed with 10 μL of fresh prepared slurry of Affi-Gel Hz hydrazide beads (Bio-Rad) overnight at ambient temperature. The glycopeptides-captured hydrazide beads were sequentially washed with ACN/H_2O (80/20, v/v), sodium chloride (1.5 M).

The releasing of the captured glycopeptides was performed through the following two protocols. First, as in the conventional hydrazide chemistry method, the beads were mixed with the lowest volume of NH_4HCO_3 buffer (10 mM, pH 8.0) consisting of 500U PNGase F enzymes (New England Biolabs) with subsequent incubation of 16 h at 37 °C. This treatment would release the deglycopeptides which left a 0.9858 Da mass labeling on the previously glycosylation site and would make it possible for identification of glycosylation sites by MS (the chemical deamidation of unmodified asparagine and glutamine also occurred randomly, which may create false positive identifications). The supernatant was collected and then the beads were washed with 100 μL of 100 mM ammonium bicarbonate twice. The supernatants were combined, desalted, and lyophilized for MS analysis. Second, in this work, the beads was first resuspended in 200 μL of 200 mM hydroxylamine buffer containing 100 mM aniline (pH 5.0) and incubated 10 h at 37 °C to cleave the hydrazone bond between the former oxidized glycopeptides and the hydrazide beads. The release glycopeptides was collected and the beads were washed with 100 μL of 100 mM ammonium bicarbonate twice. The elution of the peptides were carefully collected and mixed. After lyophilization, 100 μL of NH_4HCO_3 buffer (10 mM, pH 8.0) with 500 U PNGase F enzymes was added and shook at 37 °C for 16 h for glycopeptide deglycosylation.

Glycosylation Site and Glycoprotein Identification. The protocol of LC−MS/MS analysis was found in the Supporting Information. MaxQuant (version 1.3.0.05) was used to search all of the obtained MS/MS spectra with a composite database published by the International Protein Index (version, IPI mouse 3.87).[30] Only one fixed modification that is carbamidomethylation on cysteine (Cys, + 57.0215 Da) was set for all of the searches. Some variable modifications were set as our previous work: deamidation on asparagine and glutamine (Asp and Asn, + 0.9858 Da), oxidation on methionine (Met, + 15.9949 Da), cysteine (Cys, + 15.9949 Da), and tryptophan (Trp, + 15.9949, + 31.9898 Da).[29] As performed in our previous work, dimethylation on lysine Lys and peptide N-termini was also set as a single-plex label. The proteolytic enzyme was set as trypsin with no more than two missed cleavage sites allowed. The mass tolerance of the precursor ion and the fragment ion were set to 6 ppm and 0.05 Da, respectively. The false discovery rate of peptide identifications was accepted ≤0.01 for protein identification. All the identified deamidation sites according to the consensus sequence of N-glycosylation (N-X-[S/T], X can be any amino acids except proline) were verified as glycosylation sites. The identified

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glycoproteins must contain no less than one glycosylation site defined above.

RESULTS AND DISCUSSION

Capturing aldehyde containing proteins, peptides, or other biological samples by the hydrazide chemistry method via the formation of the hydrazone bond has been demonstrated to be highly specific and efficient. Under the catalysis of aniline, the hydrazone bond could even be efficiently formed in physiological conditions (pH 7.4). As hydrazone bond is formed through the reaction between the hydrazide group and aldehyde on oxidized glycopeptides, it is evident that it could also be cleaved through the transamination reaction.[31] It is well recognized that oxime formation has a larger equilibrium constant than hydrazone formation, thus the presence of high concentration aniline and hydroxylamine would make the hydrazone formation equilibrium move toward hydrazone bond cleavage and oxime formation. In detail, addition of aniline catalyzes the hydrolysis of hydrazone bond to hydrazide and intermediate imine, and then the hydroxylamine reacts with the generated imine very quickly which resulted in the complete cleavage of hydrazone bonds to form oximes.[32] It has been demonstrated that this method is highly efficient, approximately 80% of the hydrazone bonds could be cleaved in 3 h and the full cleavage could be achieved in 10 h.[33] We anticipate that combination of the hydrazone bond formation and the reversible cleavage technique together can highlight a more significant implementation prospect of hydrazide chemistry. Theoretically, this method can be used to covalently immobilize proteins or other biological samples onto solid surfaces with high specificity, followed by releasing them under mild conditions without sample denaturation. However, because the N-glycopeptides captured on the hydrazide beads could be cleaved by PNGase F, the cleavage of the hydrazone bond to release the glycopeptides has never been considered for N-glycoproteomics analysis.

In this study, we presented a HAPD strategy by exploiting this chemical cleavage approach as shown in Figure 1. Before the oxidization of glycopeptides, the N-terminal primary amines were first blocked by the peptide N-terminal protection (PNP) strategy.[29] This is because the hydrazide chemistry based solid-phase extraction of the N-linked glycopeptides technique failed to identify N-glycopeptides with the N-terminal serine/threonine (Ser/Thr) residue when the N-terminal amines were not blocked.[29] A side reaction of forming aldehyde groups could be generated on the vicinal amino alcohol groups of the N-terminal Ser/Thr residue during the periodate oxidation procedure.[34,35] Thus, these oxidized N-terminal Ser/Thr peptides can be covalently coupled to hydrazide beads through formation of hydrazone bonds and cannot be released by PNGase F which is specific for the cleavage of the N-glycosidic bond. Theoretically these peptides could be released by the chemical cleavage method proposed in this study and thus the identification of N-terminal Ser/Thr N-glycopeptides would not be a problem anymore. However, it should also be noted that release of tremendous nonglycosylated N-terminal Ser/Thr peptides would seriously reduce the enrichment specificity and affect the identification efficiency of the enriched deglycopeptides. Thus, the PNP strategy which blocked the peptide N-termini with dimethyl labeling to prevent the oxidation of N-terminal serine/threonine was applied to eliminate such interference. After blocking the peptide N-terminal amines, the same method as to the conventional hydrazide chemistry

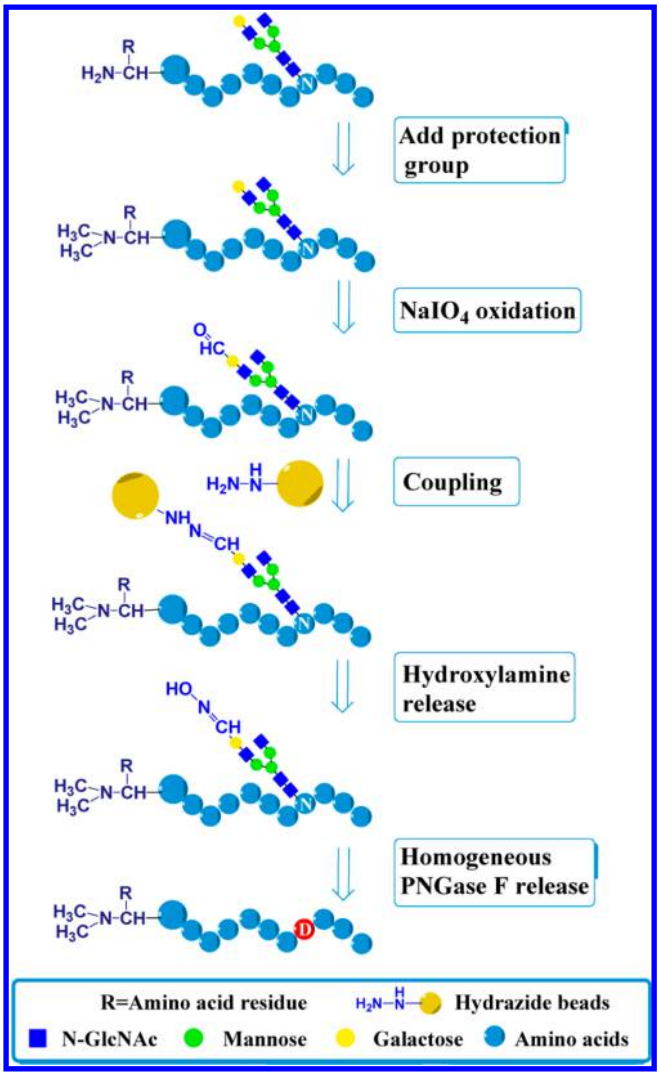

Figure 1. Efficient release of peptide moieties from hydrazide beads by a hydroxylamine assisted PNGase F deglycosylation (HAPD) strategy.

method was utilized to capture glycopeptides.[21,27] The glycopeptides captured on the hydrazide beads were first released into solution by incubating the beads with hydroxylamine solution to cleave the hydrazone bonds, which were then subjected to the PNGase F for deglycosylation in free solution prior to LC−MS/MS analysis. In this way the deglycosylation of glycopeptides in the hydrazide chemistry method was also performed in the homogeneous condition which could allow the enzyme to freely access the glycosidic bonds to realize highly efficient deglycosylation.

We first evaluated the performance of the HAPD strategy using standard glycoprotein. In brief, two aliquots of IgG digests (5 μg) were labeled with light (L, adding $(CH_2)_2$, + 28 Da) and heavy (H, adding $(CD_2)_2$, + 32 Da) dimethyl labeling, respectively. The light and heavy labeled samples were oxidized and enriched through the same hydrazide chemistry protocol, while the releasing of the captured glycopeptides was performed differently. More specifically, the light labeled one was released directly by the PNGase F to obtain the deglycopeptides and the heavy one was treated with HAPD strategy. Then light and heavy labeled samples were combined and detected by MALDI TOF/TOF 5800. The release efficiency of these two methods was compared by determining

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the intensity of these two isotope peaks of the light and heavy labeled samples. The tryptic deglycopeptides of IgG were R. EEQFN$_{de}$STFR.V (1158 Da, IgG2) and R. EEQYN$_{de}$STFR.V (1190 Da, IgG1) (where de denotes the deamidated asparagine). The peptides' mass shifted to 1186/1190 and 1218/1222 Da ($[M + H]^+$), after dimethyl heavy/light labeling (+28/32 Da), respectively. As shown in Figure 2A, the intensity

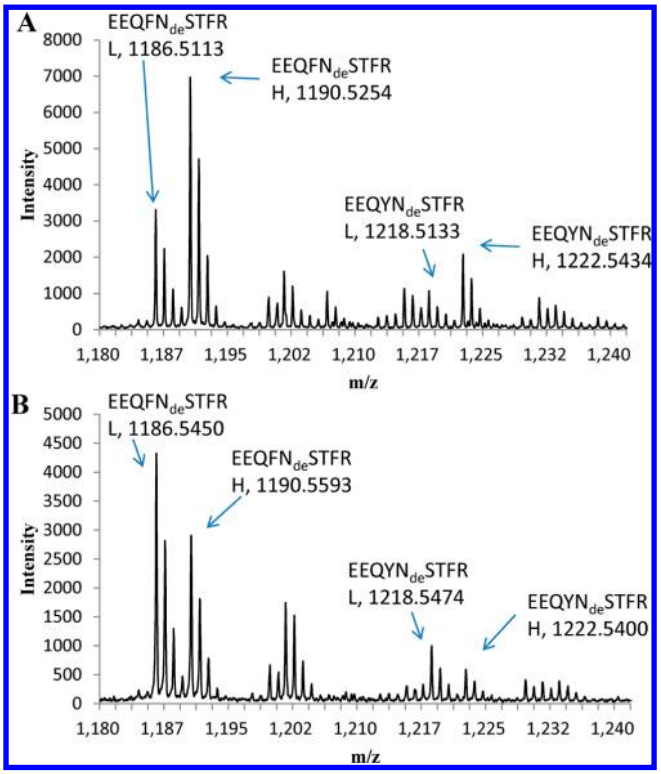

Figure 2. HAPD strategy enabled efficient release of peptide moieties. Two aliquots of IgG digest were labeled with light (L, adding $(CH_2)_2$, + 28 Da) and heavy (H, adding $(CD_2)_2$, + 32 Da) dimethyl label, respectively, before the hydrazide chemistry enrichment. (A) The light labeled one was performed by conventional PNGase F deglycosylation and the heavy one was treated with hydroxylamine assisted deglycosylation; (B) the label order was reversed.

of both the heavy labeled deglycopeptides which represent the HAPD strategy is higher than that of the light labeled deglycopeptides representing the conventional release method. The intensity ratio (H/L) of the two deglycopeptides is 2.11 ± 0.01 and 1.97 ± 0.12, respectively (Table S1), both of which demonstrated the higher release efficiency of the HAPD strategy. The isotope labeling reversed experiment showed the similar result (Figure 2B) and the intensity ratio (L/H) of the two deglycopeptides is 1.39 ± 0.09 and 1.47 ± 0.20, respectively (Table S1). Clearly, the HAPD strategy indeed improved the yield of deglycopeptides.

We then investigated the performance of HAPD strategy for the mouse liver N-glycoproteome analysis. For comparison, two approaches, the new HAPD strategy and the conventional method, were applied to release the former oxidized glycopeptides captured by the hydrazide chemistry method from the same amount of test sample (50 μg of mouse liver proteins), separately. For each method, three parallel capturing and releasing experiments were performed and the enriched deglycopeptides were analyzed by 1D LC−MS/MS, respectively. All of the experimental parameters were the same except for the glycopeptide releasing methods. As shown in Figure 3A, 642 glycosylation sites were averagely identified in the HAPD

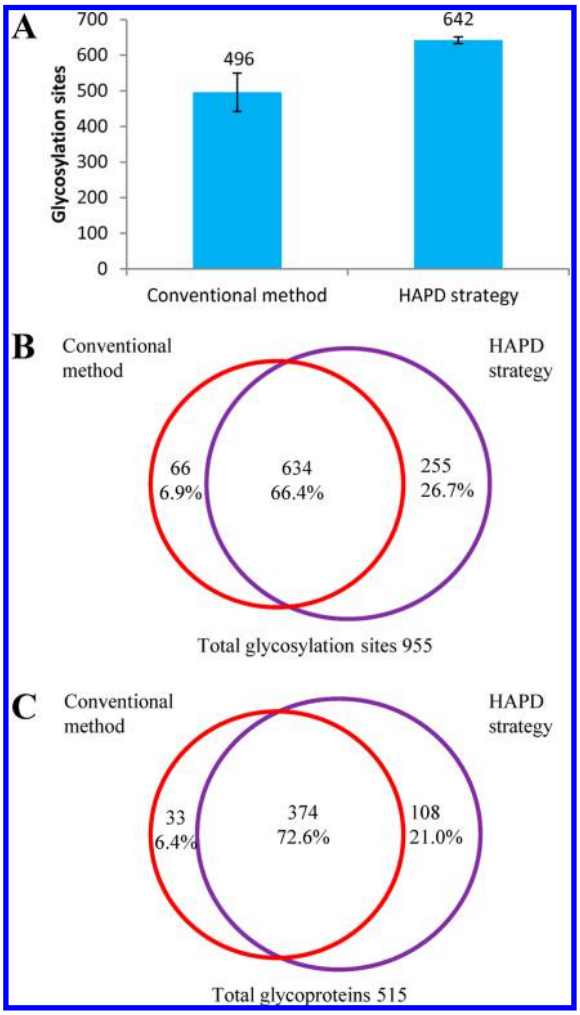

Figure 3. Experimental results of mouse liver N-glycosylation identification by the HAPD strategy and conventional PNGase F deglycosylation methods. (A) Comparison of N-glycosylation sites identified from the HAPD strategy and conventional method (data were averaged from three replicate experiments and error bars represent the standard deviation); (B) overlap of the glycosylation sites; and (C) overlap of glycoproteins identified from the two methods.

strategy, while only 496 glycosylation sites were averagely identified in the conventional method (Table S5). As shown in Figure S1, both the HAPD strategy and conventional method showed good glycoproteome mapping reproducibility. Also combination of the three replicate experiments' results together, 889 glycosylation sites corresponding to 482 glycoproteins were obtained from the HAPD strategy, while 700 glycosylation sites attributing to 407 glycoproteins were identified in the conventional method (Tables S2, S4, and S5). The new HAPD strategy yields 27% more glycosylation site identifications than the conventional release method does.

We also compared the overlaps of the identified glycosylation sites and glycoproteins from the two methods. Altogether, 955 N-glycosylation sites corresponding to 515 glycoproteins were identified and 634 (66.4%) of the sites were identified in both methods (Figure 3B,C). The results also showed that only 66 (6.9%) N-glycosylation sites were newly identified by the

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conventional method while 255 (26.7%) N-glycosylation sites were uniquely covered by the HAPD strategy. As to the proteins, the newly identified glycoproteins was 33 (6.4%) and 108 (21.0%) corresponding to the conventional and HAPD method, respectively. As most of (90%) the glycosylation sites and glycoproteins from the conventional method were also included in the HA treated method (Figure 3C), we could concluded that the HAPD strategy has higher efficiency in glycosylation identification. As to the deglycopeptide identification, the improvement of the new strategy was consistent with the glycosylation site identification. Averagely 34.9% more deglycopeptides were identified (Figure 4A, Tables S2 and S5).

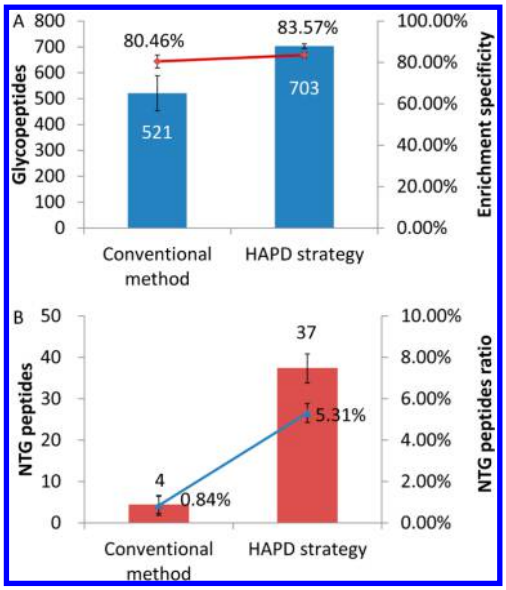

Figure 4. Comparison of all glycopeptides (A) and N-terminal glycosylated peptides (NTG) glycopeptides (B) identified from the HAPD strategy and conventional method. (Data were averaged from three replicate experiments, and error bars represent the standard deviation.)

It is worth mentioning that the enrichment specificity of the conventional method and the HAPD strategy was both above 80%, demonstrating the exclusive advantage of the hydrazide chemistry-based glycopeptide enrichment method.

Except for the general improvement of the glycosylation site and deglycopeptide identification by the HAPD strategy, we also found an interesting improvement in the identification of N-terminal glycosylated peptides which having glycosylation Asn at the peptide N-terminus. The linkage between the N-linked oligosaccharide chain and the N-terminal glycosylated Asn residue is difficult to be released by PNGase F because the enzyme does not well recognize these glycopeptides and the enzyme–substrate complexes are not very stable.[36,37] When the N-terminal glycosylated peptides were captured by the hydrazide beads, they are more difficult to be cleaved by PNGase F due to the enzyme having steric hindrance to access the bonds in the heterogeneous condition. We counted the number of N-terminal glycosylated peptides in all of the identified glycopeptides in both the conventional method and the HAPD strategy and found that on average only 4 N-terminal glycosylated peptides were identified in the conventional method and the ratio (the number of N-terminal glycosylated peptides compared to the total number of identified glycopeptides) was only 0.84 ± 0.48%. While 37 N-terminal glycosylated peptides were on average covered in the HAPD strategy and the ratio (ditto) reached 5.31 ± 0.46% (Figure 4B). We also determined the ratio of N-glycopeptides with glycosylation sites at other positions (Figure 5 and Table S3). Except for position 1, the ratio was increased slightly from 9.03 ± 1.32% to 11.00 ± 1.30%. As all other positions' *p*-values obtained from the *t* test of the ratios of the two methods was above 0.01, they do not have a significant difference (Table S3). This result demonstrated that the increase of N-terminal glycosylated peptide identification is specific and that it is closely related to whether the N-terminal glycosylated peptides are free or immobile, while the improvement of glycopeptides with glycosylation sites at other positions is general. This improvement well confirmed our hypothesis that performing the PNGase F treatment in the homogeneous condition outperforms doing that in heterogeneous conditions.

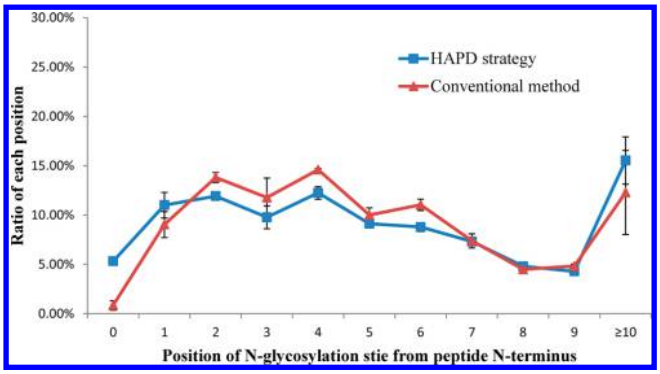

Figure 5. Ratios of N-glycopeptides with glycosylation sites located at different position from the peptide N-terminus, i.e., position 0 means the N-terminal glycosylated peptides.

CONCLUSION

In this study, we developed a HAPD strategy to efficiently release glycopeptides for the hydrazide chemistry based solid-phase extraction of N-linked glycopeptide method for glycoproteomics analysis. In the first time, hydroxylamine was used to release the former oxidized intact N-glycopeptides through transamination and then PNGase F deglycosylation was performed in the free solution, which could avoid the inefficiency of the enzymatic reaction in heterogeneous conditions. This HAPD strategy allowed for the mapping of 889 N-glycosylation sites in mouse liver by three replicate 1D LC–MS analyses. This number is 27% more than those obtained from the parallel experiments using the conventional hydrazide chemistry method. Interestingly, the identification of N-terminal glycosylated peptides was improved significantly. These improvements well demonstrated that performing the PNGase F treatment in the homogeneous condition could yield better recovery than doing that in heterogeneous conditions. We also envisage that combining the hydrazone bond formation and reversible cleavage technique together will have more applications in a variety of affinity purifications but particularly in proteomic studies where the capture, release, and analysis of protein post-translational modifications are involved.

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■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.5b02669.

Additional experimental details and results (PDF)

Glycosylation site and glycoprotein identifications comparing the HAPD and conventional methods (XLSX)

Deglycopeptide identifications of the HAPD and conventional methods (XLSX)

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Author Contributions

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Notes

The authors declare no competing financial interest.

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