Study on behaviors and performances of universal $N$-glycopeptide enrichment methods†

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Glycosylation is a crucial process in protein biosynthesis. However, the analysis of glycopeptides through MS remains challenging due to the microheterogeneity and macroheterogeneity of the glycoprotein. Selective enrichment of glycopeptides from complex samples prior to MS analysis is essential for successful glycoproteome research. In this work, we systematically investigated the behaviors and performances of boronic acid chemistry, ZIC-HILIC, and PGC of glycopeptide enrichment to promote understanding of these methods. We also optimized boronic acid chemistry and ZIC-HILIC enrichment methods and applied them to enrich glycopeptides from mouse liver. The intact $N$-glycopeptides were interpreted using the in-house analysis software pGlyco 2.0. We found that boronic acid chemistry in this study preferred to capture glycopeptides with high mannose glycans, ZIC-HILIC enriched most $N$-glycopeptides and did not show significant preference during enrichment and PGC was not suitable for separating glycopeptides with a long amino acid sequence. We performed a detailed study on the behaviors and performances of boronic acid chemistry, ZIC-HILIC, and PGC enrichment methods and provide a better understanding of enrichment methods for further glycoproteomics research.

1. Introduction

Post-translational modifications (PTMs) play an essential role in protein biosynthesis which affects the protein activity and function. 1,2 Glycosylation is one of the most abundant, important and complicated PTMs of proteins involved in many physiological functions and processes. 3–5 $N$-Glycosylation is currently the most widely studied protein glycosylation, and the most common research strategy is based on mass spectrometry (MS). However, the inherent low abundance of glycopeptides, the complexity of the glycan structure and the ion suppression of non-glycopeptides make it a big challenge to directly analyze the protein glycosylation through MS. 6,7 Therefore, it is necessary to enrich glycopeptides before MS analysis.

Numerous enrichment methods have emerged as powerful tools to capture the glycoproteins or glycopeptides. Lectin affinity chromatography, chemistry methods including hydrazide chemistry and boronic acid chemistry, titanium dioxide ($\text{TiO}_2$), hydrophilic interaction chromatography (HILIC) and porous graphitized carbon (PGC) are popular enrichment methods. 6,8 Among these methods, hydrazide chemistry is not suitable for enriching intact glycopeptides, because it involves lengthy reaction processes including the periodate oxidation reaction and formation of hydrazine bonds, and then enriched glycopeptides have to be deglycosylated prior to MS analysis. 9,10 Lectin affinity chromatography based on recognition of lectins and specific carbohydrates is not comprehensive. 11–13 Titanium dioxide based on coordination interaction between the metal ion (Ti) and negatively charged peptides is a biased enrichment method. 14,15 Boronic acid chemistry, HILIC, and PGC are generally considered to be unbiased and universal methods for intact glycopeptide enrichment. Boronic acid chemistry forms a reversible five- or six-membered cyclic ester between $\text{cis}$-diol-containing groups and boronic acid. 16 HILIC is considered as a mixed-mode mechanism, mainly based on hydrophilic partitioning and other interactions such as electrostatic interactions, dipole interactions, adsorption, hydrogen bonding and so on. 17–20 PGC retains very polar analytes that cannot be trapped by RP, so it has been used for glycopeptide separation. 21–23

There are some studies on the kinetic and thermodynamic mechanisms of boronic acid chemistry, HILIC, and PGC. 18,24–28 These studies investigated the specific recognition of monosaccharides/polysaccharides of boronic acid chemistry, and the retention of saccharides or peptides on HILIC or...
PGC respectively. Traditional glycoproteomics strategies study either glycosylation sites or glycan compositions, which could not obtain complete information about the protein N-glycosylation at the same time.\textsuperscript{29–31} On the other hand, many studies are based on the analyses of identification of glycosylation sites or the structure of glycans. Few studies considered the glycopeptide as a whole object of study. The glycopeptides consist of glycans and peptides, which are divergent in the physicochemical properties. Enrichment efficacy of the intact glycopeptides with various methods needs elaborate studies. Our laboratory has established an efficient strategy to identify the intact N-glycopeptide reported in ref. 32 and 33. In this work, we focused on the characteristics of the universal enrichment methods for intact glycopeptides, provided the comprehensive understanding of enrichment methods in enrichment performances and optimized the methods.

2. Experimental

Protein samples

Standard proteins. Asialofetuin from fetal calf serum (ASF), Peroxidase from horseradish (HRP), IgG from human serum (IgG) and Ribonuclease B from bovine pancreas (RNB) were purchased from Sigma-Aldrich (USA).

Mouse liver tissues from adult male C57BL/6 mice (3–12 months) were purchased from SLRC LABORATORY ANIMAL (Shanghai, China). The tissues were homogenized in a lysis buffer (4% SDS, 0.1 M Tris-HCl pH 7.6) by using a high-throughput tissue grinding machine (ONEBIO, Shanghai, China). The homogenates were sonicated at 65 Hz for 60 s and then clarified by centrifugation at 16 000g for 40 min. Protein concentration was determined by using BCA (Pierce, Rockford, IL). Extracted proteins were stored at \(-80 ^\circ\text{C}\) until use.

Protein digestion

The ASF and HRP were dissolved in 50 mM NH\textsubscript{4}HCO\textsubscript{3} solution, respectively, and then heated at 100 \(^{\circ}\text{C}\) for 10 min to denature the proteins. Trypsin was then added to the solution at an enzyme/substrate ratio of 1 : 40 (w/w) at 37 \(^{\circ}\text{C}\) with overnight shaking. The IgG and RNB were denatured by dissolving in 8 M urea solution, respectively. The solution was then clarified by centrifugation at 16 000g for further analysis.

Glycopeptide enrichment

Boronic acid enrichment method. Digested peptides were dissolved in binding buffers, and incubated for 1 h with rotation with Boronate Gel (BIO-RAD, USA). Then a 5\% formic acid solution and a solution containing acetonitrile: H\textsubscript{2}O:TFA at 50:49:1 were used to elute glycopeptides for 30 min, respectively. Here, we examined the effects of the binding buffer type; ammonium formate (HCOONH\textsubscript{4}) buffer, ammonium acetate (CH\textsubscript{3}COONH\textsubscript{2}) buffer and ammonium bicarbonate (NH\textsubscript{4}HCO\textsubscript{3}) buffer were evaluated for glycopeptide enrichment. The effects of the concentration of salt and pH were studied in ammonium bicarbonate buffer on the enrichment of standard samples. The detailed reaction conditions are listed in ESI Table S1.\textsuperscript{†}

ZIC-HILIC. The digestions were dissolved in a loading buffer and then loaded onto micro-columns containing ZIC-HILIC packing particles (5 \(\mu\)m, Welch, China) after equilibration with the loading buffer. After being washed with loading buffer, the retained analytes were eluted with an acid solution. The loading buffer is crucial for glycopeptide enrichment for the ZIC-HILIC method. Then we investigated the effects of the ion-pairing reagent (TFA), the presence of salt in buffer (with/without added 20 mM ABC) and the concentration of acetonitrile (ACN) on loading buffer. The detailed reaction conditions are listed in ESI Table S2.\textsuperscript{†}

PGC. The digestions were dissolved in water and then loaded onto PGC SPE columns (Thermo Fisher, USA). The column was subsequently washed three times with water, followed by elution using different concentrations of ACN solution containing 0.1\% (v/v) TFA. Different concentration fractions were collected, respectively. The detailed reaction conditions are listed in ESI Table S3.\textsuperscript{†}

MALDI-TOF-MS analysis

For the analysis of enriched glycopeptides from standard samples, 1 \(\mu\)l of the enriched glycopeptides was deposited on the target and then another 1 \(\mu\)l of matrix solution (DHB at 20 mg ml\textsuperscript{-1} in a 50\% ACN (v/v) and 0.1\% TFA (v/v) solution) was added. MALDI-TOF MS was performed in positive ion reflection mode by using a 5800 Proteomics Analyzer (AB SCIEX, USA) with the Nd-YAG laser at 355 nm, a repetition rate of 200 Hz and an acceleration voltage of 20 kV. The spectra were analyzed manually.

Nano-liquid chromatography-mass spectrometry (nano-LC–MS/MS)

Intact N-glycopeptides were analyzed on an Orbitrap Fusion Tribrid system (Thermo Fisher, USA) equipped with an EASY-
nLC-TOF1200 system (Thermo Fisher, USA) involving a reverse-phase analytical column. For one LC-MS run, samples were loaded onto a C18 column (50 cm × 75 μm i.d.) and were separated at a flow rate of 300 nL min⁻¹. Solvent A was an aqueous solution containing 0.1% formic acid. Solvent B was acetonitrile containing 0.1% formic acid. The gradient was 1 h in total for complex samples: 2% to 40% in 45 min, an increase to 90% B in 45 s, hold for another 90 s and hold for 2% B for the last 75 s. The parameters for intact glycopeptide analysis were: (1) MS: scan range (m/z) = 800–2000; resolution = 120 000; AGC target = 200 000; maximum injection time = 100 ms; included charge state = 2–6; dynamic exclusion after n times, n = 1; dynamic exclusion duration = 15 s; and each selected precursor subject to one HCD-MS/MS; (2) HCD-MS/MS: isolation window = 2; detector type = Orbitrap; resolution = 15 000; AGC target = 500 000; maximum injection time = 250 ms; collision energy = 30%; and stepped collision mode on with an energy difference of ±10% (10% as absolute value in the Orbitrap Fusion).³⁷

**Database searching**

The raw data derived from the Orbitrap Fusion were analysed by using the software pGlyco 2.0, replacing the N in the sequence N-X-[S/T] (where X is not proline) with J. The parameters are set as follows: Var Modifications, Oxidation[M], Fix Modifications, Carbamidomethyl[C], Max Miss Cleavage, 2, Precursor Tol, 5 ppm, Fragment Tol, 20 ppm, Enzyme, Trypsin_KR-C. A false discovery rate (FDR) of 2% was estimated and applied to all data. The glycan database was extracted from Glycome DB (http://www.glycome-db.org), and the total entries of N-glycan were 7884 considering NeuGc. Trypsin and protein databases with species of Mus musculus (16 711 entries) were used.

**3. Results and discussion**

We systematically investigated the behaviors and performances of boronic acid chemistry, ZIC-HILIC and PGC in enriching glycopeptides using standard glycoproteins ASF, HRP, IgG and RNB through MALDI-TOF-MS and optimized multiple experimental parameters on the three methods and applied them to enrich complex N-glycopeptides from mouse liver. The enriched N-glycopeptides were identified by using LC-MS/MS and our self-developed pGlyco2.0 software.³⁵,³¹ The workflow of this study is shown in Fig. 1.

**Investigation of enrichment method behaviors and optimization of experimental conditions with standard glycoproteins**

**Boronic acid chemistry.** The boronic acid could specifically recognize the carbohydrate by esterification interaction that occurs between the boronic acid and cis-diol-containing compounds. Under alkaline conditions, boronic acid hydrolyzes and goes from a planar triangular configuration to a negatively charged tetrahedral configuration, which could form esters with cis-diols. This reaction is reversible and the cyclic ester could be hydrolyzed under acidic conditions without altering the glycan structure.³⁴ Therefore, it is necessary to investigate the effect of pH on the boronic acid chemistry method when enriching glycopeptides. Springsteen et al. used the ARS system to evaluate the binding constants between boronic acid and cis-diol-containing compounds.³⁵ Their data showed that buffer types and concentration had effects on binding constants. Taken together, we investigated buffer types and buffer concentration as well as the effect of pH on glycopeptide enrichment.

**The effect of buffer types.** To investigate the effect of buffer types on enrichment, we compared three kinds of buffer types including ammonium formate, ammonium acetate, and ammonium bicarbonate solutions (50 mM pH 10), respectively. The number of enriched N-glycopeptides and the intensity ratio of selected glycopeptides versus non-glycopeptides (from the same spectrum, the same below) were used as the final evaluation index. As shown in ESI Fig. 1A, we found that there were no significant differences among the three kinds of the buffer in intact glycopeptide enrichment for most standard glycoproteins. Although it was reported that the buffer types would affect the binding constant between monosaccharides and small molecule diol compounds,³⁵ our data showed that the influence of buffer types was not apparent on the enrichment of intact glycopeptides in the boronic acid enrichment method. Considering the enrichment efficacy and the signal stability, ammonium bicarbonate solution was chosen as a follow-up experimental solution.

**The effect of buffer concentration.** Then we investigated the effect of buffer concentration on boronic acid enrichment. We compared 10 mM, 20 mM, 50 mM, 100 mM and 200 mM ammonium bicarbonate solution (pH 10). The intensity of spectra, the number of enriched glycopeptides, and the intensity ratio of glycopeptides versus non-glycopeptides at different concentrations of ammonium bicarbonate solution were considered for enrichment efficacy. It is suggested that buffer concentration would affect the specificity and selectivity of boronic acid enrichment of glycopeptides. And the signal response in subsequent mass spectrometry analysis was suppressed by the high-concentration buffer.

In ESI Fig. S1B, it is clearly showed that the intensity of the spectrum exhibited a downtrend as the salt concentration increased, and the signals of the analytes were almost undetectable when the solution concentration was 200 mM except for RNB samples (data were not shown). For ASF and HRP, the largest numbers of enriched N-glycopeptides were obtained at 50 mM ammonium bicarbonate. For IgG and RNB, enrichment efficacy did not change with the concentration of buffer (ESI Fig. S1C). The increase of the salt concentration generally works for suppression of the electrostatic repulsion effect.³⁶-³⁸ However, the high salt concentration is adverse to the MS signal intensity as well as stability, shown in ESI Fig. S1C. The intensity ratio of glycopeptides versus non-glycopeptides showed large fluctuations at high salt concentrations. In order to obtain the effective and stable enrichment and to
ensure the MS response, we selected 50 mM ammonium bicarbonate as the buffer concentration.

The effect of buffer pH. Finally, we investigated the effect of pH on glycopeptide enrichment. We used the number of enriched N-glycopeptides and the intensity of representative N-glycopeptide peaks to evaluate the impact.

The influence of pH on the enrichment efficacy was significant, while the response of different glycopeptides to pH varied (Fig. 2). For ASF and HRP, the largest number of enriched glycopeptides was obtained at pH 10. The number of enriched IgG glycopeptides did not change as the pH increased. The glycopeptides of RNB were not enriched at pH 7 and 8. Up to pH 10 and 11, there were five glycopeptides enriched. Extreme pH would harm the chromatography packing, so in the subsequent study, pH 10 was chosen for boronic acid enrichment.

We observed the response of different representative glycopeptides according to the changed pH. For ASF, three N-glycopeptides with the same oligosaccharide composition and different peptide sequences showed different performance as the pH increased (Fig. 2A). For RNB, three N-glycopeptides with a similar oligosaccharide composition and the same peptide sequences showed similar trends according to the changed pH (Fig. 2D). The three glycopeptides of HRP and IgG with different oligosaccharide compositions and peptide sequences exhibited totally different performance from each other.

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**Fig. 1** Workflow of the study.
other (Fig. 2B and C). These results showed that pH had a significant but complicated impact on the boronic acid chemistry enrichment method, which depended on both the peptide sequence and oligosaccharide composition of an individual glycopeptide. (Detailed N-glycopeptides composition could be seen in Tables S4–S7†.)

For the boronic acid method, we only identified three glycopeptides from IgG. The number of identified glycopeptides was much smaller than that in other methods in this study and other groups’ previous work.39 We suspected that it might be due to electrostatic interactions between negatively charged groups during the boronic acid enrichment. The boronic acid captured the cis-diol-containing compounds under alkaline conditions, which led to the electrostatic repulsion between boronic acid and negatively charged glycopeptides.16 Glycopeptides of IgG contained some negatively charged analytes, which formed strong electrostatic repulsion with boronic acid. This result indicated that boronic acid might have a discrimination effect on the sialic acid-rich glycopeptides. According to the above series of condition experiments, the mechanism of the boronic acid enrichment method based on the reversible esterification reaction was affected by the electrostatic repulsion effect, resulting in a discrimination effect on the sialic acid-rich glycopeptides, and the pH had a significant but complicated impact. The optimized experimental conditions of binding buffer were 50 mM ammonium bicarbonate solution at pH 10.

ZIC-HILIC. It has been reported that ZIC-HILIC has a mixed-mode mechanism when separating and enriching glycopeptides. In addition to hydrophilic partitioning, there also are electrostatic interactions, dipole interactions, adsorption, and hydrogen bonding.18 In this experiment, we used water-acetonitrile (ACN) as the loading buffer. It is reported that the use of an ion-pairing reagent could increase the retention of analytes and the distinction between non-glycopeptides and glycopeptides.20 The use of salt could reduce the electrostatic interactions. The ACN concentration would greatly affect the retention of glycopeptides due to hydrophilic partitioning.18 Thus, we investigated the effects of the use of the ion-pairing reagent, the presence of salt during enrichment and the concentration of ACN on ZIC-HILIC enrichment. The number of enriched N-glycopeptides and the intensity of selected glycopeptides were used as the evaluation index. (In some spectra there were not enough non-glycopeptides, so we didn’t use the intensity ratio of glycopeptides versus non-glycopeptides as the evaluation index.)
The effect of ion-pairing reagent. TFA is a commonly used reagent in glycopeptide enrichment, which is not only an ion-pairing reagent but also a strong acid reagent. In order to investigate the effect of TFA on enrichment, we compared the different concentration of TFA in 80% ACN solution during enrichment and using FA as the control group. We found that TFA could increase the retention of analytes (Fig. 3A and B).

We used the 80% ACN solution containing 0.05%, 0.1%, 0.5%, 1.0%, 3.0%, and 5% TFA as the loading buffer. For the FA control group, an 80% ACN solution containing 0.5%, 1.0%, 3.0%, 5.0%, 8.0% and 10% FA was used in order to keep the pH similar to the TFA group. According to Fig. 3A, the black line showed that the FA group identified only a few N-glycopeptides without salt and the best enrichment was obtained at 3% FA. The addition of ABC benefited the glycopeptide enrichment in the FA system, while the increased acidity showed no apparent effect on enrichment. For the TFA group shown in Fig. 3B, the increase of TFA concentration without salt improved the enrichment efficacy and the best enrichment condition was 5% TFA (the black line). These phenomena were also shown on other standard samples in this study (ESI Fig. S3†). The different enriching efficacy trends as the FA and TFA concentration increased indicated that the ion-pairing effect favored glycopeptide enrichment rather than the acid effect.

The effect of salt. The salt could shield the charged group to reduce electrostatic interaction.19 So it is general to use salt to reduce the electrostatic interactions during the enrichment.40 In this study, we added 20 mM NH₄HCO₃ (ABC) to loading buffer to evaluate the effect of salt on the ZIC-HILIC enrichment method.

In Fig. 3A, the gray line showed that the use of salt could increase the retention of glycopeptides in the FA group. When adding 20 mM ABC (the gray line), more glycopeptides could be identified than the non-salt group (the black line). But for the TFA group (Fig. 3B), there was a totally different enrichment efficacy trend. When adding 20 mM ABC to the TFA group, the line of enriched glycopeptides declined after a slight increase as well as other standard samples (Fig. 3B, the gray line and ESI Fig. S3†). It seemed that the salt limited the use of TFA under high concentrations of TFA.

During the ZIC-HILIC enrichment, TFA as an ion-pairing reagent could combine charged non-glycopeptides to increase the hydrophobicity of charged non-glycopeptides. Glycopeptides containing multiple hydrophilic groups (–OH)
would not be affected as much as non-glycopeptides. Thus, the use of TFA could benefit the glycopeptide enrichment. While the presence of salt in the loading buffer yielded charged ions during the enrichment, which would combine with TFA to limit the ion-pairing effect. In this respect, the presence of salt did hinder the effect of TFA. However, each standard sample obtained the best enrichment efficacy when using 80% ACN loading buffer containing 0.1% TFA and 20 mM ABC. These revealed that the salt probably might also have other effects on ZIC-HILIC enrichment. During the enrichment, the loading buffer contained a high level of ACN, which might make the salt tend to be rich in the water layer, resulting in an increase in the thickness of water layer. This would bring a strong retention of hydrophilic analytes. Thus, these two effects were taken together, the interaction between salt and ion-pairing reagent and the effect of salt on the thickness of the water layer, using 80% ACN loading buffer containing 0.1% TFA and 20 mM ABC as the loading buffer could also obtain high enrichment efficacy. And this conclusion could apply to all standard samples in this study (Fig. 3C and D).

**The effect of ACN concentration.** The hydrophilic partitioning occurs between the more hydrophobic mobile phase and the immobilized water-rich layer. Thus, the concentration of ACN was an essential factor for enrichment. When the ACN concentration was low, the glycopeptides could not be retained by the column.

According to Table S2, when the ACN concentration reached 90%, the signals of glycopeptides were suppressed by non-glycopeptides (ESI Fig. S4†), which might be due to the high-level ACN. The high-level ACN could induce the adsorption interaction, which is beneficial for hydrophobic analytes, such as non-glycopeptides. So ACN concentration was better between 80% and 90%, too high and too low ACN concentration were not good for enrichment.

In this part, we considered the effect of loading buffer on the ZIC-HILIC enrichment method, including the use of ion-pairing reagent (TFA), the presence of salt and the ACN concentration in loading buffer. TFA and salt had the complex interactions during the enrichment. Accounting for these factors, the optimized experimental conditions of loading buffers were: 0.1% TFA with 20 mM ABC in 80% ACN solution and 5% TFA without added salt in 80% ACN solution.

**PGC.** PGC is a novel carbon material, which can retain highly polar compounds that are not retained in the traditional reverse phase. In recent years, PGC is often combined with MS for oligosaccharide analysis, particularly for isomeric structure separation. The retention mechanisms of PGC are supposed to be various (dipole, hydrophobic, charge induce, dispersion etc.) and the conclusion is controversial. In this study, we tried to use PGC to separate standard glycoproteins by the gradient elution method. We loaded standard glycoproteins to the PGC column and used the different concentration of ACN to elute the retained analytes and then collected the fractions for analysis by MALDI-TOF-MS, respectively.

We counted the number of identified N-glycopeptides and non-glycopeptides in different fractions, as shown in Fig. 4 and the mass spectra could be seen in ESI Fig. S5† (Because different fractions contained different glycopeptides and non-glycopeptides, we did not count the ratio of glycopeptides versus non-glycopeptides.) We found that the distribution of glycopeptides from different samples in the fractions varied, and co-elution of glycopeptides and non-glycopeptides occurred in most samples, except RNB. The retentions of different glycopeptides were discrepant. Glycopeptides of RNB could be eluted completely by 20% ACN, while those of ASF and IgG started eluting at 30% ACN, the majority of glycopeptides eluted by 80% ACN. For HRP, glycopeptides started eluting at 20% ACN but did not elute completely until 80% ACN. We also found that some glycopeptides could not be eluted from PGC, such as VVHAVEVALATFNAESN#GSYLQVLVEISR-[Hex]6[HexNAc]5 (m/z 5094) of ASF, and LYN#FSNTQGLPDPTLN#TTYLQTLR-[Xyl][Hex]3[Fuc][HexNAc]2 + [Xyl][Hex]3[Fuc][HexNAc]2 (m/z 4983) of HRP. This indicated that PGC would strongly retain some analytes. It is consistent with previous reports that PGC is a strong adsorbent, and elution of large glycopeptides may be difficult. It seems that PGC is not an appropriate method for comprehensive glycopeptide separation. Then we did not do further study. Some successful work has been reported about using PGC to separate glycopeptides based on nonspecific digestion with pronase, which was able to generate short peptide sequences. In this work, the trypsin digested ASF, HRP, and IgG, yielded glycopeptides with a long amino acid sequence; the elution of glycopeptides was difficult. RNB yielded short peptide sequences containing 6 amino acids (SRNLTK), resulting in successful elution. It is clear that PGC was inappropriate for glycopeptides with a long amino acid sequence. So we did not choose PGC for glycopeptide enrichment from mouse liver.

**The enrichment efficacy of complex proteins from mouse liver.** We then used mouse liver as complex samples to evaluate
and compare the enrichment performance of the boronic acid method and the ZIC-HILIC method. After enrichment, analytes were directly injected into a LC-MS without PNGase F digestion and the data were interpreted by using pGlyco 2.0 at the intact N-glycopeptide level.

The experimental conditions of boronic acid chemistry were pH 10, 50 mM ammonium bicarbonate solution as the binding buffer, a 3% formic acid solution and a solution containing acetonitrile: H₂O : TFA at 50 : 49 : 1 as the eluent. For ZIC-HILIC, 80% ACN aqueous solution containing TFA as loading buffer: (i) 5% TFA without adding salt defined as ZIC-HILIC-N (ii) 0.1% TFA with 20 mM ABC defined as ZIC-HILIC-S. Then 1% TFA aqueous solution was used as the eluent for both experiments.

In Fig. 5A, 1352 intact N-glycopeptides and 467 glycosites corresponding to 301 N-glycoproteins were identified, of which 340 N-glycopeptides and 172 glycosites corresponding to 135 N-glycoproteins were identified by the boronic acid method, 990 N-glycopeptides and 366 glycosites corresponding to 246 N-glycoproteins were identified by ZIC-HILIC-N, and 1093 N-glycopeptides and 396 glycosites corresponding to 256 N-glycoproteins by ZIC-HILIC-S. There were 75 non-redundant glycans identified on N-glycopeptides, among which 24 glycans were found in the boronic acid method, 58 in ZIC-HILIC-N and 68 in ZIC-HILIC-S, in Fig. 5A.

The boronic acid method identified the least amount of 340 glycopeptides, accounting for 27.5% of the 1352 N-glycopeptides. Only 46 unique N-glycopeptides were identi-
fied by the boronic acid method. The specificity of the boronic acid method was nearly 31% (average of three technical replicates). (The specificity of the method herein is obtained by dividing the number of intact glycopeptides' spectra by the total number of identified spectra.) ZIC-HILIC-N and ZIC-HILIC-S identified 1306 intact glycopeptides covering 96.6% of the total identification. The ZIC-HILIC-S identified 1093 glycopeptides accounting for 80.8% of total number contributed 287 unique N-glycopeptides and the ZIC-HILIC-N identified 990 glycopeptides accounting for 73.2% of total number contributed 198 unique ones. Approximately 30% of the glycopeptides were uniquely identified by ZIC-HILIC-N or ZIC-HILIC-S. The use of an ion-pairing reagent or salt would have a complementary impact on the enrichment due to different modes of action. The specificities of ZIC-HILIC-N and ZIC-HILIC-S were 25% and 28%, respectively. According to our data, the total amount of boronic acid method identification was not as high as that of ZIC-HILIC methods, but the boronic acid method was more specific than ZIC-HILIC methods. This meant that the ZIC-HILIC method was a large-scale enrichment method, while the boronic acid method was more specific than ZIC-HILIC.

The software has carried out the classification statistics of the glycan composition. It divides glycan composition into Hex/HexNAc/Ac/Gc/Fuc and counts the number of different glycan types (in Fig. 5B). It is worth noting that more than 96% N-glycopeptides identified by the boronic acid method were high mannose type, while only one glycopeptide contained sialic acid. However it is generally considered that the glycopeptide containing sialic acid had low ionization

Fig. 6  Functional annotation of identified N-glycoproteins.
efficiency during MS analysis. This phenomenon was not observed in ZIC-HILIC-N and ZIC-HILIC-S thus indicating that the boronic acid method had the preference during the enrichment (Fig. 5B). ZIC-HILIC-N identified 69.7% N-glycopeptides containing high-mannose type and nearly 19% containing sialic acid. ZIC-HILIC-S identified 67.2% N-glycopeptides containing high-mannose type and nearly 23% N-glycopeptides containing sialic acid. Compared with the boronic acid method, ZIC-HILIC is more universal.

We analyzed the distribution of the peptide length, glycan length, the mass ratio of glycans versus peptides and the isoelectric point (pI) of peptides to analyze the intact glycopeptide properties (Fig. 5C). (The value of pI was calculated using the tool in the ExPASy bioinformatics resources portal (http://web.expasy.org/compute_pi/).) Glycopeptides identified by ZIC-HILIC-N and ZIC-HILIC-S were very similar, in all aspects. The boronic acid method was slightly different from those ZIC-HILIC methods. In detail, the distribution of the peptide length showed that the most identified N-glycopeptides contained 6–9 amino acids for all the methods. The distribution of the glycan length also showed similar results to the peptide length. Most N-glycopeptides contained 9–11 glycans and the majority of glycan lengths were less than 13. For the distribution of the mass ratio of glycans versus peptides, the distribution of the boronic acid method differed from ZIC-HILIC-N and ZIC-HILIC-S. The ranges of mass ratios of ZIC-HILIC methods were larger than the boronic acid method. Most of the N-glycopeptides identified by ZIC-HILIC had mass ratios between 0.6 and 1.8. The highest N-glycopeptide mass ratio identified by the boronic acid method was 1.2–1.5. The mass ratio here was used to roughly measure the hydrophobicity of glycopeptides. The statistical results showed that the glycopeptides enriched by ZIC-HILIC were more hydrophobic than those enriched by the boronic acid method. We suspected that the boronic acid method based on esterification interaction required the structure of the reactant. So the distribution of the boronic acid method was different from ZIC-HILIC. The distribution of peptide pI values showed that the boronic acid method preferred the glycopeptides with pI between 8–10, which was consistent with the mechanism of the boronic acid enrichment method based on the reversible esterification reaction. For ZIC-HILIC-N and ZIC-HILIC-S, the majority of glycopeptides had a pI between 4–6 and 8–10. The data of glycopeptides shown in Fig. 5 was identified in more than one time within the three technical replicates. We also used glycopeptides identified in more than 2 of the 3 replicates in each method to make the comparison (ESI Fig. S8†), which was basically the same with the comparison in Fig. 5. Then we compared the unique glycopeptides enriched by ZIC-HILIC-N and ZIC-HILIC-S, in ESI Fig. S9.† It is suggested that ZIC-HILIC-N and ZIC-HILIC-S had different preferences in the glycan length.

Characterization of N-glycoproteins in the mouse liver. We used the DAVID tool (http://david.abcc.ncifcrf.gov/) to analyze the characters of identified N-glycoproteins. The information of sub-cellular localization, biological processes and molecular functions of the identified glycoproteins were obtained. In Fig. 6, N-glycoproteins are shown to be mainly localized in the membrane, extracellular exosomes and as integral components of the membrane. Other cellular components such as endoplasmic reticulum, intracellular member-bound organelle, and Golgi apparatus were related to the ER and Golgi complex, and the lysosome was related to the process of hydrolysis. The most abundant biological processes were transport, proteolysis, metabolic process, cell adhesion, and lipid metabolic process. Many molecular functions that were supposed to be performed by N-glycoproteins were identified, the top three were hydrolase activity, protein binding, and peptidase activity. Detailed information about the top ten enriched N-glycoproteins is shown in Fig. 6.

Conclusions

We systematically investigated the behaviors and performances of boronic acid, ZIC-HILIC, and PGC in the enrichment of glycopeptides at the standard protein level and complex biological samples. According to our results, PGC was not suitable for enriching trypsin-digested glycopeptides due to the co-elution of glycopeptides and non-glycopeptides, as well as the difficulty in elution of the glycopeptides. ZIC-HILIC methods showed superior sensitivity and universality to the boronic acid method when enriching glycopeptides. The combination of different enrichments is a good and potential solution for glycoproteomics study. In light of the above discussion, our study provided insight into commonly used enrichment methods and contributed to further biological function research of N-glycoproteins.

Conflicts of interest

There are no conflicts to declare.

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Notes and references
