Centrifugation Assisted Microreactor Enables Facile Integration of Trypsin Digestion, Hydrophilic Interaction Chromatography Enrichment, and On-Column Deglycosylation for Rapid and Sensitive N-Glycoproteome Analysis

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Supporting Information

ABSTRACT: Sample handling procedures including protein digestion, glycopeptide enrichment, and deglycosylation have significant impact on the performance of glycoproteome analysis. Several glycoproteomic analysis systems were developed to integrate some of these sample preparation procedures. However, no microsystem integrates all of these procedures together. In this work, we developed a glycoproteomic microreactor enabling seamless integration of all these procedures. In this reactor, trypsin digestion was accelerated by adding acetonitrile to 80%, and after acidification of protein digest by trifluoroacetic acid (TFA), the following hydrophilic interaction chromatography (HILIC) enrichment and deglycosylation were sequentially performed without any desalting, lyophilization, or buffer exchange steps. The total processing time could be as short as 1.5 h. The detection limit of human IgG as low as 30 fmol was also achieved. When applied to human serum glycoproteome analysis, a total number of 92, 178, and 221 unique N-glycosylation sites were identified from three replicate analyses of 10 nL, 100 nL, and 1 μL of human serum, respectively. It was demonstrated that the glycoproteomic microreactor based method had very high sensitivity and was well suited for glycoproteome analysis of minute protein samples.

Protein glycosylation is one of the most important post-translational modifications and plays a vital role in a myriad of crucial biological processes, such as protein folding, cell–cell interaction, molecular recognition, and so on. The aberrant glycosylation is a basic feature of oncogenesis and progression of numerous diseases. Most of the clinically used cancer biomarkers are glycoproteins, such as α-fetoprotein (AFP) in hepatocellular carcinoma, cancer antigen (CA) 125 in ovarian cancer, and prostate-specific antigen (PSA) in prostate cancer. Therefore, glycoproteome of various body fluids, such as serum, cerebrospinal fluid, saliva, etc., have been frequently analyzed to discover potential biomarkers of cancers and other diseases. Among them, serum is the most frequently used specimen due to its clinical importance in disease diagnosis and therapeutic monitoring.

Though 50% of all human proteins are considered to be glycosylated, the low abundance of glycopeptides as well as low ionization efficiency in mass spectrometry (MS) makes the enrichment step indispensable for MS-based glycoprotein identification. Fortunately, several effective methods including hydrazide chemistry, lectin affinity chromatography, boronate affinity chromatography, and hydrophilic interaction chromatography (HILIC) have been developed for the enrichment of glycoproteins and glycopeptides. Among them, HILIC enrichment methods have drawn increasing attention these years. A variety of hydrophilic matrixes including sepharose, cellulose, Polyhydroxyethyl A, ZIC-HILIC, and click maltose-HILIC have been developed for glycopeptide enrichment. It was demonstrated that the click maltose-HILIC has good efficiency and selectivity for enrichment of glycopeptides.

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Usually, precious biological samples of specific diseases are available with a very limited amount, thus the strategies for microscale glycoproteome analysis are urgently needed. Several novel glycoproteome analysis systems have been established recently. Zhou et al.28 developed a glycoproteomic reactor that enabled the integration of digestion and deglycosylation. Their approach led to the identification of 82 glycopeptides corresponding to 41 glycoproteins using 5 μL of human serum. However, the enrichment process was not integrated in the reactor, and using Con A-agarose column for glycoprotein enrichment compromised the performance of glycoproteomic analysis. Qu et al. introduced a sample pretreatment system by integration of the steps of glycopeptide enrichment and deglycosylation.26 This system was applied to analysis of rat brain glycoproteome, and 196 N-linked glycosylation sites corresponding to 120 glycoproteins were identified using 6 μg of protein sample. The main drawback of this system lies in that protein digestion step was not integrated. Thus the labor-intensive desalting, lyophilization steps, as well as the overnight incubation for protein digestion were performed separately, which may cause significant sample loss and an analyzing throughput decrease. Mann et al. developed a novel system named filter-aided sample preparation (FASP) for analysis of mouse tissue N-glycoproteome.16,29 This system integrated all steps of digestion, enrichment, and deglycosylation; however, it is not suitable for microscale glycoproteome analysis due to the big size of the filter units used in their system.

In this work, a centrifugation assisted microreactor allowing facile integration of acetonitrile improved digestion (AID) of proteins, click maltose HILIC enrichment, and deglycosylation of glycopeptides was established for glycoproteome analysis of microliter samples. Briefly, protein sample was first digested with 80% acetonitrile in the reactor, then the digest was acidified by TFA and the glycopeptides were enriched with HILIC beads under centrifugation. Finally, the enriched glycopeptides were eluted and deglycosylated with a solution of 20 mM NH4HCO3 containing 100 U PNGase F for LC−MS/MS analysis. The use of 80% acetonitrile for trypsin digestion not only significantly reduces the digestion time but also facilitates the downstream processing steps. The whole process can be completed within 1.5 h. To the best of our knowledge, this is the first glycoproteomic microreactor integrating all three steps of digestion, enrichment, and deglycosylation.

■ EXPERIMENTAL SECTION

Reagents and Materials. IgG, avidin, fetuin, α1-acid glycoprotein, serotransferrin, and trypsin were purchased from Sigma (St. Louis, MO). PNGase F was from New England Biolabs (Ipswich, MA). Chemical reagents of iodoacetamide (IAA), 1,4-dithiothreitol (DTT), and trifluoroacetic acid (TFA) were obtained from Sigma (St. Louis, MO). Formic acid (FA) was obtained from Fluka (Buches, Germany). Acetonitrile (ACN, HPLC grade) was from Merck (Darmstadt, Germany). Ammonium bicarbonate and urea were from Bio Basic Inc. (Ontario, Canada). Deionized water used in all experiments was purified with a Milli-Q system from Millipore (Milford, MA). Other chemicals were all of analytical grade. The human serum used in all the experiments was obtained from the Second Affiliated Hospital of Dalian Medical University (Dalian, China) and stored at −80 °C until usage. The utilization of human serum complied with guideline of the Ethics Committee of the Hospital. The centrifugal filter units (Amicon Ultra-0.5 mL) were purchased from Millipore (Milford, MA). GELoader tips (20 μL) were purchased from Eppendorf (Hamburg, Germany). C18 AQ beads (3 and 5 μm, 120 Å) were from Michrom BioResources (Auburn, CA). Aspire RP30 Desalting Tips were purchased from Thermo Scientific (San Jose, CA).

Preparation of HILIC-SPE Tips. HILIC SPE tips were made as previously described with minor modification.25,30 Briefly, the GELoader tip was first packed with a small piece of cotton wool.

Figure 1. (A) Seamless workflow for glycoproteome analysis and (B) photographs of HILIC tips and the constructed microreactor.
as the sieve.\(^{31}\) Then, 0.5–1 mg of click maltose HILIC beads (4 \(\mu m, 100 \text{ Å}\)) were packed into the tip by centrifugation at 4 000g for several minutes. The photographs of HILIC tips and the device for centrifugation were presented in Figure 1B. Finally, the tip was washed by 10 \(\mu L\) of \(H_2O\) and equilibrated with 10 \(\mu L\) of 80% \(ACN\) before usage.

Optimization of Enrichment Conditions. For optimization of enrichment conditions, 0.5 mg of IgG and avidin were digested and desalted using a conventional protocol as we described previously.\(^{32,33}\) After the digest was redissolved in loading buffer (0.1% FA/80% ACN, 0.1% TFA/80% ACN or 1% TFA/80% ACN), 20 \(\mu L\) of solution equivalent to 1 \(\mu g\) of protein was pipetted into a HILIC tip. After centrifuging at 4 000g for about 10 min, the HILIC tip was washed with 10 \(\mu L\) of loading buffer and then eluted with 10 \(\mu L\) of \(H_2O\). For evaluating the on-column deglycosylation performance, 1 \(\mu g\) of IgG or avidin digest was loaded onto the HILIC tip and then the enriched glycopeptides were eluted with 20 mM NH\(_4\)HCO\(_3\) containing 100 U PNGase F via centrifuging at 800–1 000g for about 30 min. The eluted intact glycopeptides and deglycosylated peptides were detected by matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI-TOF MS) directly.

Integration of Acetonitrile-Improved Digestion, Click Maltose-HILIC Enrichment, and On-Column Deglycosylation. Standard glycoprotein samples, IgG, avidin, and five-glycoprotein mixture (IgG, avidin, fetuin, \(\alpha\)-1-acid glycoprotein, and serotransferrin), were first denatured by 8 M urea/100 mM NH\(_4\)HCO\(_3\). Then 20 \(\mu g\) of the denatured samples were added to a centrifugal filter unit with 3 KDa cutoff, respectively. After reduction and alkylation,\(^{32,33}\) the samples were desalted by centrifugation for 15 min at 14 000g and washed with 400 \(\mu L\) of \(H_2O\) twice. After the above processes, about 40 \(\mu L\) of liquid was left in the filter unit. For the human serum sample, the same procedures described above were performed.

All the following procedures were performed in the microreactor (Figure 1B). A volume of 2 \(\mu L\) (1 \(\mu g\)) alkylated standard glycoprotein sample (IgG, avidin, or five-glycoprotein mixture) or 2 \(\mu L\) of the alkylated serum solution (equivalent to 10 \(nL\), 100 \(nL\), or 1 \(\mu L\) human serum) and 0.5 \(\mu L\) of trypsin were added to a HILIC tip at a protein to trypsin ratio of 10:1, followed by adding 10 \(\mu L\) of ACN. The tip was held at 37 °C for 30 min. After digestion, 10 \(\mu L\) of 80% ACN/2% TFA was added to acidify the sample in the tip. Then, the HILIC tip was centrifuged at 4 000g for about 10 min, followed by washing with 20 \(\mu L\) of 80% ACN/1% TFA and 20 \(\mu L\) of 80% ACN for 10 min, sequentially. The enriched glycopeptides were then deglycosylated and eluted with 20 \(\mu L\) of 20 mM NH\(_4\)HCO\(_3\) containing 100 U PNGase F to a microtube by centrifuging very slowly at a speed of 1 000g for 30 min. The eluted peptides were finally detected directly by MALDI-TOF MS or analyzed by liquid chromatography–tandem mass spectrometry (LC–MS/MS). The whole processes including digestion, enrichment, and deglycosylation cost less than 1.5 h for standard glycoproteins. For serum analysis, the digestion and deglycosylation were both extended to 60 min. So, the whole processes will last for about 2.5 h.

RESULTS AND DISCUSSION

Design of a Glycoproteomic Microreactor Enabling Integration of Protein Digestion, Glycopeptide Enrichment, and Deglycosylation. The sample handling procedure for glycoproteomic analysis usually includes three steps, i.e., protein digestion, glycopeptide enrichment, and glycopeptide deglycosylation. In conventional methods, these steps are usually performed separately, which inevitably causes sample loss due to the involvement of extra steps of desalting and lyophilization. It is reported that acetonitrile up to 80% could enhance the activity of several enzymes including trypsin and endoproteinase Glu-C.\(^{34,35}\) Also, solution with high organic solvent content is a perfect loading buffer for HILIC enrichment of glycopeptides. These facts arouse us to perform trypsin digestion in buffer with...
high acetonitrile concentration followed by HILIC enrichment. A designed workflow for analysis of glycoproteome was given in Figure 1A. After trypsin digestion in 80% acetonitrile, the protein digest was acidified and directly loaded to HILIC column without desalting. Considering the compatibility with downstream processing, low concentration volatile buffer, i.e., 20 mM NH$_4$HCO$_3$ containing PNGase F, was used as the elution buffer. The addition of PNGase F in the elution buffer made the procedures of elution and enzymatic deglycosylation simpler. This volatile buffer provided alkaline pH for PNGase F digestion and had minimal interference for MS analysis. For detection of intact glycopeptides by MALDI MS, the deglycosylation step could be omitted. The above workflow does not need any desalting steps and has significant potential for glycoproteome analysis. However, to make the above workflow seamless and sensitive, a microreactor allowing easy processing of the above steps should be constructed.

The photographs for the constructed microreactor were presented in Figure 1B. It consisted of one HILIC tip and two centrifugal tubes. The HILIC tip was used for glycopeptides capture. The other two tubes were used to fasten the HILIC tip for centrifugation. The centrifugation with the device facilitated the sample loading and elution procedures. In the beginning, the protein digestion in 80% acetonitrile was performed in the HILIC tip on top of the HILIC beads. After digestion, the digest was acidified by FA or TFA. The resulting solution was forced to flow through the HILIC beads by centrifugation, which enabled the selective capture of glycopeptides. Then, the captured glycopeptides were eluted directly with H$_2$O or eluted with 20 mM NH$_4$HCO$_3$ containing 100 U PNGase F for ~30 min.

**Optimization of Glycopeptide Enrichment and On-Column Deglycosylation.** The performance of the microreactor largely depends on the performance of the HILIC beads. Click maltose HILIC beads were selected in this study due to their better glycopeptide enrichment performance compared with commercially available HILIC materials such as Sepharose CL-6B. To further improve the performance of glycopeptide enrichment, loading buffer was optimized. ACN–H$_2$O–FA (80:20:0.1) was used as the loading buffer in previous studies. However, it was reported that TFA could act as an ion-pairing reagent to improve the efficiency for glycopeptide enrichment by HILIC. Therefore, the enrichment performance by using loading buffers with and without TFA was compared. To enable easy comparison, tryptic digest of a single glycoprotein (avidin or IgG) was applied as a standard sample, and the enriched peptides were detected by MALDI-TOF MS. The tryptic digest was first dissolved in the loading buffer and then loaded onto the click maltose-HILIC tip. After washing with the loading buffer, the captured glycopeptides were eluted with

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**Figure 2.** MALDI-TOF mass spectra of 1 μg of chicken avidin digest (A) before enrichment and (B–D) after enrichment by click maltose-HILIC tips with different loading buffers. The enriched glycopeptides were marked with "∗". (E) Spectrum after on-column deglycosylation with 20 μL of 20 mM NH$_4$HCO$_3$ containing 100 U PNGase F for ~30 min.
10 μL of H2O. As shown in Figure 2B, a number of nonglycopeptides from avidin could still be detected after enrichment using 0.1% FA/80% ACN as the loading buffer. However, when using loading buffers with TFA, i.e., 0.1% TFA/80% ACN and 1% TFA/80% ACN, almost only glycopeptide peaks could be observed as shown in Figure 2C,D. Similar results for human IgG digest were also observed as shown in Figure S1 (Supporting Information). As 1% TFA/80% ACN showed the best enrichment performance, it was adopted as the loading buffer in the following enrichment experiments.

The deglycosylation of glycopeptides before LC−MS/MS analysis is typically required for identification of N-glycopeptides. This step is usually performed offline in solution, which suffers from some drawbacks such as long incubation time, manual manipulation, and so on. Qu et al. used a PNGase F immobilized enzymatic reactor (IMER) for online deglycosylation after HILIC enrichment.26 However, a strong cation exchange (SCX) precolumn was required between the HILIC column and IMER for buffer exchange. This may lead to the loss of some glycopeptides due to the weak binding of sialic acid-containing glycopeptides and the strong binding of some other glycopeptides on the SCX column. To solve this problem, we developed an on-column deglycosylation strategy by eluting the enriched glycopeptides very slowly with buffer containing PNGase F. Thus the enzymatic deglycosylation began to occur on the column and was completed in the eluate during the elution process under centrifugation. Moreover, the deglycosylated peptides can be directly eluted to a microtube for autosampling. Avidin digest was utilized for evaluation of on-column deglycosylation. It can be seen from Figure 2E that only the deamidated peptide WTNDLSN#MTIGAVNSR was detected after on-column deglycosylation, which demonstrated the high efficiency of deglycosylation. Human IgG digest was also used for evaluation of on-column deglycosylation and similar results were obtained (Figure S1E in the Supporting Information).

**Evaluation of the Performance of Glycoproteomic Microreactor Using Standard Glycoproteins.** Recently, several types of reactors have been established for glycoproteomic analysis of human serum and mouse brain with high detection sensitivity.26,28 However, no microreactor integrating all the processing steps of protein digestion, glycopeptide enrichment, and deglycosylation together was reported. Usually, biological samples were first digested using a conventional digestion approach, and then a small amount of digest was taken to the reactors for glycopeptide enrichment. The desalting and lyophilization steps are always necessary after conventional digestion, which will unavoidably cause significant sample loss. Thus the sensitivity of glycoproteome analysis with these reactors will decrease significantly when a minute amount of starting material is used.

In our microreactor, no desalting and lyophilization step is required, which makes the whole process faster and more efficient. The total processing time could be reduced to only 1.5 h, in contrast to 2 days or more for conventional methods.
Standard glycoprotein samples with 1 μg of human IgG, chicken avidin, and five-glycoprotein mixture were separately applied to evaluate the performance of the established microreactor for glycoprotein analysis. After digestion and HILIC enrichment, the intact glycopeptides of IgG and avidin were analyzed by MALDI directly. The obtained mass spectra with the peaks marked with probable glycans attached to peptides were shown in Figure 3A,C. Furthermore, the obtained mass spectra of the deglycosylated peptides of those glycopeptides after on-column deglycosylation were shown in Figure 3B,D, respectively. It can

Table 1. Glycopeptides Identified from 1 μg of the Five-Glycoprotein Mixture by Using the Microreactor Based Method

<table>
<thead>
<tr>
<th>Protein name</th>
<th>Glycopeptide sequences</th>
<th>Site position</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serotransferrin</td>
<td>CGLVPVLAENYNS</td>
<td>432</td>
<td>219.88</td>
</tr>
<tr>
<td></td>
<td>CQQHLSGSNVTDCSGMCFR</td>
<td>630</td>
<td>253.93</td>
</tr>
<tr>
<td>Fetuin A</td>
<td>LCPCPAPLAPNDSR</td>
<td>156</td>
<td>202.7</td>
</tr>
<tr>
<td></td>
<td>KLCPCPAPLAPNDSR</td>
<td>156</td>
<td>229.79</td>
</tr>
<tr>
<td></td>
<td>PTVEHYEIDTLETGHVDPTPLANS</td>
<td>99</td>
<td>195.83</td>
</tr>
<tr>
<td>Fetuin B</td>
<td>IFYLPAYNCTLRPVSQAIRMTCPDCPSTSPYDLSNPR</td>
<td>137</td>
<td>156.82</td>
</tr>
<tr>
<td></td>
<td>GERMNVRQPNAPS</td>
<td>271</td>
<td>114.31</td>
</tr>
<tr>
<td></td>
<td>GERMNVRQ</td>
<td>271</td>
<td>200.12</td>
</tr>
<tr>
<td>α-glycoprotein 1</td>
<td>NEYNNKS</td>
<td>56</td>
<td>161.85</td>
</tr>
<tr>
<td></td>
<td>QDQCFFNTLYNLVRQ</td>
<td>93</td>
<td>384.28</td>
</tr>
<tr>
<td></td>
<td>QDQCFFNTLYNLVRQRENGTSR</td>
<td>93/103</td>
<td>125.21</td>
</tr>
<tr>
<td>α-glycoprotein 2</td>
<td>NEEYNKS</td>
<td>56</td>
<td>161.85</td>
</tr>
<tr>
<td></td>
<td>QNCFYSSYLNVRQ</td>
<td>93</td>
<td>248.13</td>
</tr>
<tr>
<td></td>
<td>QNCFYSSYLNVRQRENGTVSR</td>
<td>93/103</td>
<td>84.12</td>
</tr>
<tr>
<td>IgG 1</td>
<td>EECVNSTYR</td>
<td>180</td>
<td>123.03</td>
</tr>
<tr>
<td>IgG 2</td>
<td>EECVNSTFR</td>
<td>176</td>
<td>145.42</td>
</tr>
<tr>
<td>IgG 3</td>
<td>EECVNSTFR</td>
<td>227</td>
<td>133.06</td>
</tr>
<tr>
<td>IgG 4</td>
<td>EECVNSTFR</td>
<td>177</td>
<td>175.4</td>
</tr>
<tr>
<td>Avidin</td>
<td>WTNLGSNNMTGAVNRS</td>
<td>41</td>
<td>446.81</td>
</tr>
<tr>
<td></td>
<td>CSLTGKWTNLDGSNNMTGAVNRS</td>
<td>41</td>
<td>175.65</td>
</tr>
</tbody>
</table>

“*The glycosylation sites were marked in bold red.”
be seen that the enriched glycopeptides were fully deglycosylated and only the deamidated peptides were detected.

The reproducibility of the microreactor based analysis was evaluated using human IgG, and highly reproducible results were obtained (Figure S2 in the Supporting Information). The detection sensitivity of the microreactor for analysis of human IgG was also investigated, and the obtained results were presented in Figure 4. It was observed that the glycopeptides from 30 fmol of human IgG were well detected by MALDI-TOF MS. For comparison, conventional method was also applied as we described in the Experimental Section, and it was observed that the detection limit was about 150 fmol (Figure S3 in Supporting Information). This is mainly because significant sample loss occurred during the desalting and lyophilization processes in conventional methods.

Then, the reactor was applied to analyze a semicomplex sample containing five standard glycoproteins. The obtained results of LC–MS/MS analyses were listed in Table 1. It can be seen that 16 out of 20 N-glycosylation sites annotated in the SwissProt Database were identified from only 1 μg of the five-glycoprotein mixture. The MS/MS spectra of the identified glycopeptides were presented in Figure S4 (Supporting Information). The above results indicated that our newly developed microreactor by integrating the protein digestion, glycopeptide enrichment, and deglycosylation together improved the detection sensitivity of glycopeptides greatly, which will be a very useful technology for glycoproteome analysis of minute amounts of starting sample (etc., less than 1 μL).

**N-Glycoproteome Analysis of Human Serum Using Minute Sample.** Finally, the microreactor was applied to glycoproteome analysis of human serum. A different amount of denatured human serum samples were subjected to glycoproteome analysis with the microreactor approach by integrating protein digestion, glycopeptide enrichment, and deglycosylation together. Finally, 92, 178, and 221 unique N-glycosylation sites were identified from three parallel LC–MS/MS analyses of 10 nL, 100 nL, and 1 μL human serum, respectively. The number of corresponding proteins were 58, 80, and 105, respectively. All the results were summarized in the Supporting Information. Apparently, the more serum sample added, the more glycosylation sites identified. However, protein precipitation was observed during digestion of 1 μL of serum in 80% ACN because the protein concentration is too high (about 6 mg/mL). Therefore, this microreactor is more suitable to process serum samples less than a microliter.

Although identification of 303 glycoproteins and 639 N-glycosylation sites from human plasma, representing the largest number of glycoproteome identification that has been reported,56 800 μL of plasma was used in that case. Also extensive sample preparation including depletion of six high-abundance proteins and fractionation of the plasma sample into 30 SCX fractions were performed. Such a strategy is very tedious and has low detection sensitivity, which is applicable only for a large amount of samples. There are many other reports for glycoproteome analysis by using 15–100 μL of human serum or plasma,15,28,37,58 and usually less than 100 glycoproteins were identified. However, in our case a larger number of glycopeptidies and glycoproteins were identified within about 2.5 h of sample preparation time by using a much less amount of human serum (10 nL–1 μL of serum), which indicated that our microreactor based method by integration of the acetonitrile improved digestion, click maltose-HILIC enrichment, and on-column deglycosylation together is of ultrahigh detection sensitivity and is well suited for microscale glycoproteome analysis of minute protein samples.

**CONCLUSIONS**

A seamless workflow was established for microscale glycoproteome analysis by using an in-house built microreactor. In this reactor, protein digestion, glycopeptide enrichment, and glycopeptide deglycosylation are performed sequentially without any desalting, lyophilization, or buffer exchange steps, and the total processing time could be shortened to about 2–3 h. The excellent performance of this microreactor was demonstrated by using standard protein samples as well as human serum sample. Using this system, 178 unique N-glycosylation sites were identified with only 100 nL of serum sample. The microreactor based method has high sensitivity and efficiency and was well suited for microscale glycoproteome analysis of minute biological samples.

**ASSOCIATED CONTENT**

## Supporting Information

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

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**Notes**

The authors declare no competing financial interest.

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