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Online 2D-LC-MS/MS Platform for Analysis of Glycated Proteome

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Abstract

Glycated proteins are emerging as good indicators for diabetes and age related diseases. However, the platform for analysis of glycated proteome has been relatively less well established. We here introduce an online 2D-LC-HCD-MS/MS platform for comprehensive glycated peptide quantification. This platform includes a boronate affinity column in the first dimension for enrichment, reversed phase nanoLC column in the second dimension for separation, a benchtop Orbitrap mass spectrometer with HCD-MS/MS for peptide sequencing, and MaxQuant bioinformatics tool for identification and quantification of glycated peptides. This online 2D-LC-HCD-MS/MS platform has high enrichment efficiency with 85% of identified peptides in the enriched fraction as glycated, high sensitivity for detection of glycated peptides with LOD and LOQ at 1.2 and 2.4 pg, respectively, and high reproducibility with inter-day CVs < 20% for 80% of the glycated peptides. The number of glycated peptides quantified in *in-vitro* glycated human plasma increased more than three-fold using this platform in comparison to that obtained using 1D-LC-HCD-MS/MS platform without boronate affinity enrichment. Application of this online platform to human plasma identified 376 glycated peptides from 10 µg of protein digests. This highly sensitive and reproducible online 2D platform is promising for glycated protein analysis of complex clinical samples.

Abstract

Graphical Abstract

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ASSOCIATED CONTENT

Supporting Information

Methods for protein digestion and desalting, boronate column packing, additional Figures(PDF) and Tables (Excel) are provided as Supporting Information.

The Supporting Information is available free of charge on the ACS Publications website.

The authors declare no competing financial interest.

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Protein glycation is a non-enzymatic reaction between reducing sugar and the primary amine groups of amino acids. It first produces labile Schiff base and then slowly isomerizes to relatively stable Amadori compound modified proteins before further oxidation to advanced glycation end products (AGEs).¹ As precursor to AGEs, Amadori compound resulted glycation have recently attracted increased attention in proteomics research due to their clinical relevance to diabetes and diabetic complications.²⁻⁴ However, an efficient platform for comprehensive analysis of glycated proteome has not been well established.

Glycated proteins can be analyzed by targeted techniques including enzyme-linked immunoassay⁵ and western blot,⁶ but only a few proteins can be measured at one time and it highly depends on the availability of antibodies. Proteomic level analysis of glycated peptides relies on tandem mass spectrometry (MS/MS). Among the most accessible ion activation methods for analysis of Amadori compound modified peptides, collision induced dissociation (CID) generally results in abundant neutral losses and the sequence specific ions are rarely observed.⁷ Neutral loss triggered MS3 and multi-stage activation can alleviate this problem by further dissociating the neutral loss specific product ions produced during the MS2 scan;⁸ however, this is achieved on instruments with MSⁿ capability, and the additional stage of ion activation also reduces throughput of analysis. Electron transfer dissociation (ETD) is well suited for glycated peptide analysis with almost complete sequence coverage,⁷ albeit with some drawbacks in sensitivity since ETnoD products can accumulate, particularly for doubly charged precursor ions which typically require additional collision activation for their dissociation;⁹ in addition, ETD is not a technique commonly available on most mass spectrometers. Alternatively, higher energy collisional dissociation (HCD) has also been utilized to analyze glycated proteome.^{10,11} During HCD, precursor ions are accelerated to collide with neutral gas, which generates both abundant sequence-specific b and y ions and neutral losses from the Amadori compound modification. When the database search engine is capable of taking into account the signature neutral losses, it can be a powerful tool for comprehensive identification of glycated proteome, as demonstrated recently by Keilhauer et al., who identified 101 glycated peptides in normal human plasma using LC-HCD-MS/MS in combination with MaxQuant for data analysis.¹²

Due to the low abundance of *in-vivo* glycation in clinical samples, boronate affinity enrichment has been applied to selectively enrich glycated peptides for increased detection sensitivity.^{1,9,11} However, the enrichment of glycated peptides were generally performed in an offline fashion, which takes long time for sample preparation and in turn introduces variations in quantification between samples. Online 2D-LC-MS/MS system can largely

solve these problems as it has been demonstrated in protein post-translational modification (PTM) studies, such as phosphorylation,¹³ glycosylation.¹⁴ To our knowledge, little has been reported for its application in glycosylated proteome analysis, even though boronate affinity monolithic column has been used online either with capillary electrophoresis or with LC for enriching cis-diol containing metabolites.^{15,16}

In this study, we developed a sensitive and reproducible online 2D platform for glycosylated (i.e. Amadori compound modified) proteome analysis, which integrates a self-packed boronate affinity column for glycosylated peptides enrichment in the first dimension, reversed phase nanoLC column for separation of enriched peptides in the 2nd dimension, QExactive HF benchtop Orbitrap mass spectrometer with HCD-MS/MS to dissociate glycosylated peptides, and MaxQuant bioinformatics tool for glycosylated peptide identification and quantification with incorporation of Amadori compound-characteristic neutral losses. The evaluation of the performance of this online 2D-LC-HCD-MS/MS platform showed that it has high sensitivity, selectivity and reproducibility in enriching glycosylated peptides from both *in-vitro* glycosylated and normal human plasma.

EXPERIMENTAL SECTION

Chemicals and materials

All chemicals and reagents were purchased from either Sigma-Aldrich (St. Louis, MO) or Thermo Fisher Scientific (Waltham, MA), unless otherwise stated. Sequencing-grade modified trypsin was obtained from Promega (Madison, WI). The PEEK tubing (1.0 mm i.d. $\times$ 1.6 mm o.d.) for self-packed boronate affinity column was purchased from IDEX Health & Science LLC (Oak Harbor, WA). Glycogel II boronate affinity gel was manufactured by Pierce Biotech (Rockford, IL) and obtained from Dr. Bart Haigh of the Institute for Bioanalytics (Branford, CT). Standard glycosylated peptide (SGP) containing Amadori compound modification on internal lysine (GEFVW[K(D-Fructosyl)]SHK, 1278 g/mol, purity 61.7%) was synthesized by Protein Technologies Inc (Tucson, AZ). Glycation of SGP was confirmed in house with ETD-MS/MS and HCD-MS/MS as shown in Figure S1 and S2. Bovine serum albumin (BSA) was purchased from Santa Cruz Biotechnology (Dallas, TX). Human plasma samples, collected with K2-EDTA as anticoagulant and passed through 0.2 μ filter, was obtained from BioreclamationIVT (Chestertown, MD) and pooled from 3 healthy people.

Sample preparation

In-vitro glycosylated bovine serum albumin (GBSA) and *in-vitro* glycosylated human plasma (GHP) were prepared according to a previous study.⁹ Briefly, BSA (100 mg/mL) and pooled human plasma (10 mg/mL) were incubated with 1 M D-glucose in 50 mM Tris HCl buffer (pH 7.5) at 37 °C for 48 h before terminating the reaction at -20°C. Prior to protein digestion, free glucose in GBSA and GHP was removed with 3KDa Microsep™ Advance Centrifugal Filter (Thermo Fisher Scientific).

Proteins (10 mg/mL) of GBSA, GHP, and HP were denatured, alkylated and digested with trypsin according to a previous study.⁹ The final digestion mixture was desalted using C18

SPE cartridges (Biotage) and eluted peptide solutions were concentrated by speed-vac before further analysis.

To evaluate the sensitivity of online 2D-LC-MS/MS system, SGP was dissolved in water and spiked into GBSA digests (0.5 $\mu\text{g}/\mu\text{L}$) at different concentrations (ranging from 0.0012 – 0.5 $\text{ng}/\mu\text{L}$). The limits of detection (LOD) and limits of quantification (LOQ, $\text{CV} < 20\%$) was calculated following the ICH Harmonised Tripartite Guideline.¹⁷

Online 2D-LC-MS/MS system setup for enrichment and analysis of glycosylated peptides

The online 2D-LC-MS/MS system consisted of an UltiMate 3000 RSLC Nano system (including a WPS 3000 autosampler and a VWD-3400 UV detector, ThermoScientific), a self-packed boronate affinity enrichment column (1 mm $\times$ 5 cm), a C18 trap column (5 μm , 100 $\text{\AA}$ particles, 300 μm $\times$ 5 mm, ThermoScientific), and a C18 analytical column (2 μm , 100 $\text{\AA}$ particles, 75 μm $\times$ 50 cm, ThermoScientific) connected to a QExactive HF Orbitrap MS (ThermoScientific) with an EASY Spray interface (Figure 1). The boronate affinity column was packed in PEEK tubing with slurry of Glycogel II boronate affinity gel by gravity settling. Buffers (A: 50 mM ammonium acetate, pH 8.1, in water; B: 0.1 M acetic acid in water) and a stepwise gradient (0% B for 7 min; 0–100% B in 0.1 min, 100% B for 13 min; 100–0% B in 0.1 min; 0% B for 20 min) at flow rate 30 $\mu\text{L}/\text{min}$ was used to enrich glycosylated peptides. The non-glycosylated peptides were first eluted to waste (Figure 1). Then the glycosylated peptides were diverted to trap column at 15 min followed by releasing the trapped glycosylated peptides to the C18 analytical column at 19 min (Figure 1) for further LC separation and MS analysis of peptides. Mobile phases for peptide separation (A: 0.1% FA in water; B: 0.1% FA in CH_3CN) were delivered by the UltiMate 3000 RSLC Nano system. The gradient time was 90 mins (0–19 min, 4% B; 19–19.1 min, 5% B; 19.1–63 min, 35% B; 63–63.1 min 90% B; 63.1–77 min, 90% B; 77–77.1 min, 4% B; 77.1–90 min, 4% B). Eluted peptides were ionized by nano-electrospray in positive mode and analyzed by QExactive HF orbitrap MS with HCD fragmentation. MS/MS data were acquired in data dependent mode (top 15 method) with one full MS scan (resolution 60,000 at m/z 200) followed by 15 MS/MS scans (resolution 15,000 at m/z 200). Other settings for analysis include: full MS AGC target of 1e^6 , MS/MS AGC target of 1e^5 , dynamic exclusion of 20 s, and mass isolation window of 1.4 m/z , normalized collision energy (NCE) at 27%.

Protein identification and quantification

The search parameters of MaxQuant software (version 1.5.3.30)¹⁸ with Andromeda search engine were as follows: UniProt human database (12/3/2014) containing 89,734 entries; fixed modification: carbamidomethylation of cysteine (57.021464 Da); variable modifications: N-acetylation of protein N-termini (42.010565 Da) and oxidation of methionine (15.994915 Da); glycation of lysine (162.052823 Da) with different neutral losses. The minimum peptide length was set to 7 amino acids and a maximum of 2 missed cleavages was allowed for the search. Trypsin/P was selected as the specific proteolytic enzyme. The global false discovery rate (FDR) cut-off used for both peptides and proteins levels were 0.01.¹⁹ The “match between runs” option was used where specified in the text with a match time window of 0.7 min and an alignment time window of 20 min. The other parameters used were the default settings in MaxQuant for processing orbitrap-MS data.

RESULTS AND DISCUSSION

The online 2D LC-MS/MS platform for analysis of Amadori compound modified peptide included boronate affinity column for enriching glycosylated peptides in the first dimension, nanoLC for separation of glycosylated peptides in the second dimension, HCD-MS/MS for generating peptide sequence specific ions indicating glycosylation site, and MaxQuant data processing software for identification and quantification of glycosylated peptides (Figure 1). Optimization of the performance of this platform, the sensitivity, enrichment efficiency, and reproducibility in glycosylated proteome analysis, and its application to clinical sample analysis are described below.

Parameter optimization for the online 2D LC-HCD-MS/MS platform

To increase the efficiency of boronate affinity chromatography in enrichment of glycosylated peptides and its compatibility with downstream glycosylated peptide separation, we optimized the UV detection wavelength (Figure S3) and mobile phase flow rate (Figure S4A) in the first dimension separation using GBSA digests since flow rate of the mobile phase may affect the binding equilibrium between glycosylated peptide and the resin-bound boronic acid in affinity enrichment.²⁰ For the four wavelengths that we tested, 215 nm gave the highest response, but acetic acid used in the elution step introduced high background absorbance due to the –COOH group in acetic acid (Figure S3). Among the rest three, 280 nm had the relative higher response for glycosylated peptides than 254 nm and 260 nm. With respect to the flow rate, we tested flow rate at 20, 30, and 50 $\mu\text{L}/\text{min}$ for this microbore column and the back pressure was 9, 12, and 18 bar, respectively, when connected to the trap column. Although the back pressures are all under the pressure limit of resin (25 bar), flow rate at 30 $\mu\text{L}/\text{min}$ gave slightly higher (0.5%) enrichment efficiency (ratio between bound glycosylated peptides to total peptides) than the other two flow rates (Figure S4A). Therefore, wavelength at 280 nm and flow rate at 30 $\mu\text{L}/\text{min}$ were chosen for the first dimension enrichment separation.

Loading capacity of the boronate affinity column was also evaluated by plotting the peak area versus the amount of *in-vitro* GBSA digests loaded onto column (Figure S4B). The maximum loading capacity was 10 μg digested peptides with 1.1 μg being glycosylated (glycosylated peptides percentage 11%, Figure S4A), which matched well with the capacity of the downstream trap column (1.1 μg) and nanoLC column (6.7 μg) for further separation of glycosylated peptides.

With respect to identification of glycosylated peptides, MaxQuant was used with consideration of various neutral losses. Amadori compound modification is a labile PTM, as such, neutral losses of $1\text{H}_2\text{O}$, $2\text{H}_2\text{O}$, $3\text{H}_2\text{O}$, CH_6O_3 , CH_8O_4 , and $\text{C}_5\text{H}_{10}\text{O}_6$ are present under HCD conditions.^{7,12} These characteristic neutral losses can be used for improving the specificity in sequencing glycosylated peptides. Of these neutral losses configured in the Andromeda search engine of MaxQuant, $3\text{H}_2\text{O}$ neutral loss identified the highest number of glycosylated peptides and also provided the highest peptide to spectrum matching score (Figure S5). This is consistent with a recent study using HCD fragmentation for identifying glycosylated peptides.¹² Normalized collision energy (NCE) at 27% was used for fragmenting glycosylated peptides as

optimized on the online 2D-LC-HCD-MS/MS platform (data not shown), which is the same NCE as used in a previous study with the same instrument model.¹²

Sensitivity of the online 2D LC-HCD-MS/MS system

The sensitivity of online 2D system was evaluated by spiking the SGP (in the range of 0.0012 ng/μL to 5 ng/μL) into 0.5 μg/μL GBSA peptides. Each concentration was measured in triplicates. Excellent linearity was achieved from 0.0024 ng to 2.5 ng between injection amount and peak area of extracted ion chromatogram (Figure S6). LOD and LOQ of SGP achieved by our online 2D method were 1.2 pg (0.94 fmol) and 2.4 pg based on ICH criteria of CV <20%¹⁷ (Table S1). The low LOD and LOQ values suggest that this online 2D-LC-MS/MS system is sensitive to measure very low abundant (sub fmol) glycosylated peptides. In general, glycosylated proteins in human plasma had wide dynamic range, from high abundant glycosylated albumin and HbA1C down to trace amount fructoselysine-containing short-lived proteins.¹ Clinically relevant glycosylated proteins could be very low abundant in human plasma,¹ which requires a highly sensitive 2D LC-MS/MS platform as outlined here.

Efficiency of the online boronate affinity chromatography for enrichment of glycosylated peptides

Next, we evaluated the enrichment efficiency of boronate affinity chromatographic system by using *in-vitro* GHP digests. Figure 2A shows chromatograms for the enrichment and separation of glycosylated peptides. In total, 964 glycosylated peptides from 161 proteins were identified in GHP (3 replicates) using the online 2D-LC-MS/MS system (Table S2), this accounts for 85% of all identified peptides (glycosylated and nonglycosylated). By using 1D-LC-MS/MS platform (without boronate affinity enrichment) alone, only 14% peptides (293) were glycosylated peptides (Table S2). The average number of identified glycosylated peptides and proteins in 1D system and 2D system were shown in Figure 2B. It is of note that the percentage of non-glycosylated peptides (15%) in the Glycogel II resin-enriched fraction is lower than that in the TSK-gel (17.6%–26.3%).¹¹ This suggests the higher selectivity of Glycogel II resin in binding glycosylated peptides, and that the porosity, particle size as well as boronic acid loading density all affect the binding of glycosylated peptides and the overall enrichment efficiency.

The enrichment efficiency of glycosylated peptides using online 2D system with HCD fragmentation is higher than offline 2D with ETD fragmentation (76.4%).²⁰ The high enrichment efficiency of online 2D platform is likely due to the higher dissociation efficiency of HCD and the 3H₂O neutral loss configured in MaxQuant search,¹² since both of these studies used Glycogel II as the affinity enrichment resin. In addition to the enrichment efficiency, the number of identification (964 glycosylated peptides, 161 glycosylated proteins) is higher than what reported in a previous study (557 glycosylated peptides, 110 glycosylated proteins),¹¹ which also used *in-vitro* GHP but an offline 2D-LC-HCD-MS/MS with TSK-gel for enrichment and a low scan rate orbitrap instrument.¹¹ It is of note that only 10 μg sample was injected in this work whereas 800 μg sample was used in that study.¹¹ Although the number (964) of glycosylated peptides identified in this study was lower than that identified from *in-vitro* GHP reported (3841) previously,²¹ it can be attributed to the employment of in-depth immunodepletion at protein level and fractionation at peptide level

in the previous study, which can significantly increase the proteome coverage. The higher number of identified glycosylated peptides with small amount of sample injection demonstrates the successful integration of two dimensional separations in analysis of glycosylated peptides from complex samples, the high efficiency of online boronate affinity enrichment and the utility of HCD-MS/MS in glycosylated peptide sequencing.

Reproducibility of the online 2D LC-HCD-MS/MS system in quantifying glycosylated peptides

To evaluate the reproducibility of this online 2D system, we ran tryptic digests of *in-vitro* GHP sample 9 times in three consecutive days using label-free proteomics approach. In total, 1034 glycosylated peptides were identified and quantified from *in-vitro* GHP (Table S3). The quantitative reproducibility was evaluated on the quantified number of glycosylated peptides in 9 replicates (Figure 3A), very good correlation was achieved between runs (Figure 3B). Overall, around 80% of glycosylated peptides had coefficient variance (CV) between days <20% (Table S3). We also picked five glycosylated peptides with 3 orders of magnitude difference in their abundance and their quantification were highly reproducible (Figure 3C). As online 2D platform could avoid the variation caused by offline sample manipulation, it makes the quantitative comparison of glycosylated peptides in different samples more straightforward and reliable.

Application of online 2D-LC-HCD-MS/MS platform to analyze glycosylated human plasma proteome

We also applied the online 2D platform to identify and quantify glycosylated peptides in human plasma digests. In total, 376 glycosylation sites from 64 proteins were confidently identified with average location score of 0.96 and mean mass error of 0.08 ppm (Table S4). Both the location score and mass error results were better than that obtained from 1D platform.¹² Moreover, we identified more glycosylated peptides than that (101) by 1D-LC-HCD-MS/MS¹² and (113) by offline 2D with HCD and CID-MS3.¹⁰ As expected, the number of glycosylation sites in this study was lower than the 2,205 glycosylated peptides identified in pooled human plasma because immunodepletion and in-depth fractionation strategy were employed in that study.²¹ The differences in human plasma samples may also contribute to the observed difference because this study used human plasma pooled from three healthy people whereas in that study human plasma pooled from 238 patients with normal glucose tolerance and 122 patients with abnormal glucose tolerance were used.²¹ With respect to the well-studied glycosylated human serum albumin, we identified 57 lysine (K) glycosylation sites, including 55 glycosylation sites from *in-vitro* GHP and 49 glycosylation sites from human plasma (Table S5). These glycosylation sites covered all the reported lysine glycosylation sites from previous studies.^{10,12,21,22} Moreover, we also found 7 glycosylation sites, K236, K310, K456, K560, K562, K584, K588, which were not reported in either *in-vitro* glycosylated human serum albumin¹² or human plasma from both the healthy and diabetic subjects after immunodepletion and in-depth fractionation.²² Furthermore, online 2D platform gave high overlap (244 glycosylated peptides) in the quantified glycosylated peptides and good quantitative reproducibility (80% of glycosylated peptides had CV <20%, Table S4) in human plasma digests with three replicates. All these results indicate that online 2D-LC-HCD-MS/MS platform can be applied to clinical plasma samples having lower glycosylation level than that of *in-vitro* GHP.

CONCLUSIONS

To our knowledge, this is the first study to establish a highly sensitive, selective, and reproducible online 2D-LC-HCD-MS/MS platform for accurate identification and quantification of Amadori compound modified glycosylated peptides. The level of glycation and non-glycation, as measured from the UV detector in the first dimension of boronate affinity enrichment, provides a basis for more accurate normalization in label-free global proteomics. Using this platform, glycosylated peptide coverage increased more than three-fold in comparison with the same platform without online boronate affinity enrichment. In addition, it had good reproducibility in quantification with small inter-day CVs and was successfully applied to quantify glycosylated peptides from human plasma. It should be of value in quantitative glycosylated proteome analysis as the abundance and the glycation level of the proteins involved in diabetes and other age related diseases may be low, which poses a significant challenge for their accurate quantification to prove their relevance in the pathogenesis of these diseases. However, it is of note that boronate affinity enrichment only works for Amadori compound modified peptides because of its specific binding to cis-diols. Because of the lack of cis-diols in their molecular structures, the advanced glycation end products, such as carboxymethyl and methylglyoxal modified proteins, cannot be enriched with this method.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Schematic drawing of the online 2D-LC-HCD-MS/MS platform. Boronate affinity enrichment column and C18 column were used as the first and second dimensional separations. The non-glycosylated peptides (non-binding fraction) were first eluted to waste (valve A with connection in blue); the glycosylated peptides (binding fraction) were eluted from affinity column to trap column (valve A with connection in red) before eluting to C18 column. A: valve for trapping and RPLC separation of peptides; B: valve for affinity separation of glycosylated peptides.

Figure 2.

The chromatograms of boronate affinity enrichment and reversed phase LC separation of glycated peptides from *in-vitro* glycated human plasma digest (A); the average number of identified peptides and proteins, glycated peptides and proteins from three replicate runs using 1D-LC-HCD-MS/MS without boronate affinity enrichment (1D) and 2D-LC-HCD-MS/MS (2D) (B).

Figure 3.

Quantitative reproducibility of online 2D-LC-HCD-MS/MS system performed with 9 technical replicate runs of *in-vitro* glycated human plasma digests in 3 consecutive days. Number of quantified glycated peptides (A); color-coded R^2 values for the binary comparison of the glycated peptides (B); Log₂ intensity of 5 glycated peptides covering 3 orders of magnitudes across 9 replicates (C). (# means Amadori modification, GlyPep1: VFDEFK#PLVEEPQNLIK#QNCELFEQLGEYK; GlyPep2:

FNWYVDGVEVHNAK[#]TK[#]PR; GlyPep3: QLEQVIAK[#]DLLPSR; GlyPep4:
LK[#]EFGNTLEDK[#]AR; GlyPep5: SIYK[#]PGQTVK,).

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