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Glycan Analysis by Reversible Reaction to Hydrazide Beads and Mass Spectrometry

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Abstract

Investigation into glycoproteins and their associated glycans is the key to understanding the function of glycoproteins in biological pathways and disease development. Current methods for glycan analysis are generally based on multiple preparation processes to separate glycans from proteins and other molecules prior to analysis. During the multistep purification processes, glycans are continuously lost and the procedure increases the difficulty for accurate quantitative analysis of glycans. Here we describe the development of a novel technique, which uses hydrazide beads to capture glycans. It is based on the conjugation of glycans to hydrazide beads through the formation of reversible hydrazone, washing out unbound nonglycans, then releasing captured glycans by acids. The results showed that the glycans were able to be isolated from concatenate peptides by using hydrazide beads. This technique was also applied to the analysis of glycans from sera sample. The integrated capture-release on the solid-phase simplifies the procedure for glycan preparation from a complex mixture and can be a powerful tool for glycan analysis.

Protein glycosylation has been considered as one of the most significant protein modifications. It has been widely recognized that glycosylation is associated with disease progression, such as cancer,¹⁻³ heart failure,^{4,5} and other congenital disorders.^{6,7} The investigation of glycoproteins and their associated glycans is the key to understanding glycoprotein functions in biological pathways and disease development as well as biomarker discovery.⁸⁻¹² To this end, we have developed the solid-phase extraction of glycopeptides (SPEG) for capture of glycosylated peptides, which has been widely applied to both quantitative analysis of glycoproteins and identification of glycosylation sites.¹³⁻¹⁸ In this method, glycosylated peptides from digested glycoproteins are captured by using hydrazide beads after glycans on glycopeptides are oxidized. Following the removal of

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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>.

nonglycosylated peptides, these glycosylated peptides are then enzymatically released from the solid support for mass spectrometry (MS) analysis. Using this method, thousands of new N-linked glycosylation sites have been identified.¹⁹⁻²¹ However, the glycans are oxidized during the capture processes and their structures are unable to be identified.

Several methods have been developed for glycan analysis. Typically, glycans are first released from glycoproteins or glycopeptides by enzymes such as Peptide: N-Glycosidase F (PNGase F) for N-linked glycans^{22,23} or by chemical reactions, like β -elimination for O-linked glycans.^{24,25} Upon the release, glycans are desalted and purified from enzymes, chemicals, and their concatenate peptides for mass spectrometry analysis.^{26,27} Although glycans are purified by separating them from peptides and other nonglycan molecules by using a variety of methods such as affinity column,²⁶ reverse-phase high-performance liquid chromatography,²⁸ capillary electrophoresis,²⁹ hydrophilic interaction chromatography,^{30,31} or multidimensional separations,³² the major obstacle for these methods is their incapability to completely separate glycans from other species, especially from the hydrophilic peptides or salts. In terms of glycan purification, the graphite guard column is a widely used medium for glycan purification, mostly for the removal of salts and small molecules.³³⁻³⁵ However, the graphite column separates glycans and other molecules in the complex samples based on hydrophobicity; the column will also isolate the nonspecific hydrophilic species and the low molecular weight of peptides in the glycan fraction. For example, studies showed that peptides can be efficiently eluted by reverse phase high-performance liquid chromatography (RP-HPLC; Jupiter C5 reversed-phase column) using 30% acetonitrile.³⁶ The complete elution of glycans from graphite HyperSEP Hypercarb columns requires up to 50% acetonitrile.³⁴ Glycans and peptides are likely coeluted in the same fraction. As a result, the yield and specificity of glycans recovered from complex glycoprotein samples remain low. To increase the specificity and improve the overall performance of glycan analysis, a new method using chemical reaction on solid-phase is introduced in this study.

Using hydrazide coated superparamagnetic silica particles, we developed a novel and highly specific approach to isolate glycans from the peptide mixture and other contaminants by reversible reaction to hydrazide beads.³⁷ The reducing ends of glycans released from glycoproteins reacted to the hydrazide on the bead surface, conjugating glycans on beads. After washing the beads, the glycans were released from beads using acids and analyzed by mass spectrometry. This novel method provides the unique means to isolate glycans from other components in complex mixture for glycomics analysis and presents a potential for clinical application.

EXPERIMENTAL SECTION

Materials and Reagents

All chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless specified. Matrix assisted laser desorption/ionization (MALDI) matrix (2,5-dihydroxybenzoic acid (DHB, >99.0% purity, 30 mg/mL)) was freshly prepared in 50% methanol and 0.1% TFA solution in an amber glass vial (Tubing Vials, Fisher Scientific, Pittsburgh, PA). In-house synthesized hydrazide coated superparamagnetic silica particles (15.2 μ m in diameter) were used for the glycan capture.³⁷ Maltotetraose (DP4),

maltopentose (DP5), maltohexanose (DP6), and maltoheptaose (DP7) were from Sigma-Aldrich.

Peptide and Glycan Preparation

Two standard peptides, angiotensin I human acetate salt hydrate (AG, 1296.68 Da, mono- H^+ , 0.39 mg) and neurotensin (NT, 1672.91 Da, mono- H^+ , 0.50 mg), were dissolved in 300 μL of high-pressure liquid chromatography (HPLC) grade water. Standard glycans, Man-9 ((Man)₉(GlcNAc)₂, 1883.67 Da, 20 μg), maltopentose (DP5, 851.26 Da, mono- Na^+ , 7.2 mg), maltohexanose (DP6, 1013.31 Da, mono- Na^+ , 8.6 mg), and maltoheptaose (DP7, 1175.37 Da, mono- Na^+ , 10 mg) were dissolved in HPLC grade water, forming 0.21 mM of Man-9 glycan and 10 mM of the four DP mixture. A volume of 5 μL of serum was dissolved into 200 μL of 0.4 mM ammonium bicarbonate buffer (pH 8.0–8.3), followed by denaturation at 100 °C for 5 min. After cooling the sample to room temperature, trypsin at 500 μL of 1 $\mu\text{g}/\mu\text{L}$ was added into the serum solution and incubated at 37 °C for 16 h. The digested sample was heated at 100 °C for 5 min to deactivate trypsin before adding 10 μL of PNGase F (500 U/ μL , New England BioLabs Inc., Ipswich, MA). The sample was then incubated at 37 °C for 16 h, dried in a Speed-Vac, and resuspended in HPLC water. Before analyzing peptides and glycans from the digested solution, the sample was purified using a graphite column (Grace Davison Discovery Sciences, Milwaukee, WI) to remove the salts and other small molecules.

Glycan-Bead Conjugation

A volume of 1 mL of magnetic beads was placed in a 1.5 mL polypropylene snap-cap microcentrifuge tube (Fisher Scientific, Pittsburgh, PA). The magnetic beads were preconditioned using 1 mL of 85:15 solution of methanol–acetate buffer (150 mL of acetate buffer in 850 mL of HPLC grade methanol), repeated two times. The supernatant was then removed after the beads had stuck on the tube sidewall using a magnetic particle separator (Invitrogen Corporation, Carlsbad, CA). A volume of 5 μL of glycan and the peptide mixture was added to the beads containing 100 μL of acetate buffer and methanol. The aniline was added as a catalyst with a final concentration 10 mM to perform conjugation of glycan reducing ends to hydrazide beads.³⁸ The sample was mixed with beads in a microcentrifuge tube over a vortex mixer (VM-3000, VWR International, Radnor, PA) and placed in a microwave oven (EMS-820; Electron Microscopy Sciences, Hatfield, PA) to react for 20 min at 50 °C with the microwave oven power at 50%. To avoid overheating the sample-beads solution, a 1–2 min interval was set for every 5 min of microwave irradiation.

Glycan Release

The conjugated glycans on beads were rinsed using 1 mL of 50 mM ammonium bicarbonate (pH 8.0–8.3). Nonconjugated samples (including peptides) were washed away after five times of rinsing. Formic acid (200 μL , 10%) was added into cleaned beads, which were placed in a 60 °C oven and incubated for 60 min. After cooling to room temperature, supernatant was collected using magnetic separator and beads were rinsed two more times by 200 μL of 5% acetic acid solution. The supernatant was cleaned using a Carbohydrate Extract-Clean LC column (Grace Davison Discovery Sciences, Milwaukee, WI), which was activated by 0.1% TFA in 50% acetonitrile (3 mL, 2 $\times$) and 0.1% TFA in HPLC grade water

(3 mL, 3×) sequentially. Samples were eluted by 50% acetonitrile with 0.1% formic acid (800 μ L, 2×). After drying in Savant Speed-Vac (Thermo Scientific, Asheville, NC), HPLC grade of solution (15 μ L of 0.1% TFA in 50% methanol/DI) was added to resuspend the samples.

MALDI-MS

The 384-well spot of μ Focus MALDI plate (Hudson Surface Technology, Fort Lee, NJ) was used. This plate was treated for sample concentration on surface to improve detection sensitivity. The sample of 1.5 μ L was added onto MALDI plate and mixed with 1.5 μ L of DHB matrix (30 mg/mL DHB). The MALDI-MS was performed with Shimadzu AXIMA Resonance (Shimadzu, Columbia, MD) with positive mode.

RESULTS AND DISCUSSION

Glycan Capture Using Reversible Hydrazone Solid-Phase Extraction (rHSPE)

In order to analyze intact glycans from glycoproteins, we developed a novel method of glycan isolation using reversible hydrazone solid-phase extraction (rHSPE). The steps are described in the following (Figure 1): (1) glycan release from glycoproteins, glycans are released from denatured glycopeptides; (2) glycan conjugation to solid support, the glycans in complex mixture are then conjugated on magnetic hydrazide beads at reducing ends; (3) removal nonglycans, beads are rinsed and washed to remove other species in sample mixture; (4) glycans hydrolysis from solid support, bead–glycan conjugation are incubated in an acidic condition (pH < 3.0) and glycans are hydrolyzed from beads; (5) glycan analysis, the released glycans are collected and analyzed.

Each released glycan possesses a reducing end, which can form an aldehyde group in an acidic condition (pH 3.0–5.5). Because of their dominant ring structure, the nucleophilic reagent is used to favorably attack the ring structure, eventually forming an acyclic structure with the aldehyde group. The aldehyde then reacts with hydrazide on the solid support through the formation of hydrazone.³⁹ The glycan–hydrazide conjugation is hydrolyzed when the conjugated glycans are incubated at 60 °C in 10% formic acid solution as shown in Figure 2.

Glycan Hydrazide Conjugation and Hydrolysis

To determine the conjugation and releasing for glycans to hydrazide, we analyzed glycan conjugation and hydrolysis in solution with adipic acid dihydrazide (ADH) (Figure 3). We tested glycans before and after conjugation as well as before and after hydrolysis by MALDI-MS. Although MALDI-MS is not the best method to precisely quantify the glycan, we used the peak intensity of each compound to estimate its relative abundance. To find right pH condition for glycan conjugation, we conducted Man-9 conjugation studies at pH 7.0 and 5.0. At neutral pH conditions, less than 10% conjugation was observed by estimation of the intensity ratio between glycan and conjugation product with ADH and Man-9 at a ratio of 100:1. For the reaction at pH 5.0, the conjugation was significantly improved up to approximately 90% (Supplementary Figure 1 in the Supporting Information). The conjugation also required a catalyst.⁴⁰ For example, it was believed that formation of a

Schiff-based intermediate by aniline lowered the reaction Gibbs free energy.³⁹ We observed a high conjugation of ADH and Man-9 with addition of 10 mM aniline, and the conjugation was significantly decreased without addition of aniline (Supplementary Figure 2 in the Supporting Information). For glycan releasing, we were able to completely hydrolyze the conjugated glycan-hydrazide by adding 10% formic acid.⁴¹ Figure 3 demonstrates the complete process of glycan-hydrazide conjugation and hydrolysis. In pH 5.0 acetate buffer, the mixture of Man-9, ADH, and aniline reacted via microwave irradiation (20 min, 50% power), forming conjugation Man-9-ADH (mono- Na^+) (2062.63 Da) (Figure 3, middle spectrum). The conjugated products were then mixed with 200 μL of formic acid solution (10%, vol) and heated at 60 $^{\circ}\text{C}$ for 60 min. The full MS scan was showed that the Man-9-ADH was hydrolyzed into original Man-9 (1906.65 Da) without glycan degradation (Figure 3, insert). This reversible conjugation-hydrolysis process demonstrates the ability to use hydrazide on the solid-phase for conjugation and hydrolysis of glycans.

Solid-Phase Glycan Capture-Release

The conjugation-hydrolysis in solution is adapted to the solid-phase by conjugating glycans to hydrazide coated superparamagnetic silica particles. As shown in Figure 2, the beads coated with hydrazide on the surface can use the similar principle of reversible hydrazone formation to isolate glycans by conjugation and hydrolysis as implemented in the solution. Because hydrazide is anchored on the beads, it has advantages over the solution to separate other components such as peptides, enzymes, and chemicals that are in solution after conjugation of glycans on hydrazide beads.

Although the chemistry of hydrazide on beads is similar to that in solution, the reaction still needs to be further adapted for hydrazide-glycan conjugation on beads due to the thermodynamic (diffusion in solution vs absorption on surface) and surface morphology difference. For glycan conjugation to beads, we used 70% methanol and 30% acetate buffer (pH 5.0) because we observed that beads easily settled down in acetate buffer alone while they were uniformly suspended in the 70% methanol and acetic buffer to increase the reaction hydrodynamics. The other conjugation-hydrolysis conditions determined from in-solution used the glycan conjugation-release on beads.

The key step for high glycan recovery is to complete the conjugation reaction and keep the hydrazone stable during the washing process. In solid phase capture, the reaction involves two equilibrium steps, between the acyclic form $\rightleftharpoons$ cyclic form and between glycan-hydrazide $\rightleftharpoons$ hydrazone. Any factor that favors the formation of acyclic and hydrazone can significantly contribute to the higher yield on conjugations. It is expected that an increased amount of beads could be favorable for conjugation since it increases a higher amount of hydrazide to react with. To determine the optimal amount of hydrazide beads used to conjugate glycans, we determined different amount of hydrazide-beads, 0.1, 1, and 5 mL, necessary for the glycan capture. The results showed that 1 mL of beads has a similar recovered yield compared to 5 mL of beads for capture on glycans (Supplementary Figure 3 in the Supporting Information).

To determine the conjugation and hydrolysis conditions, we constructed a series of mixtures including standard glycans, standard peptides, mixture of standard glycans and peptides, and

mixture of complex glycans and peptides from a complex biological sample (human serum) (Table 1). A volume of 5 μL was taken from 20 μL of sample mixture (Table 1). Each sample was mixed with beads in methanol–acetic buffer solution (85:15, vol/vol). The supernatant in each step was collected and dried in a Speed-Vac at 37 °C. Beads were then immersed in 200 μL , 10% formic acid for 60 min at 60 °C. The results showed that both standard peptides were removed from the glycan–peptide mixture after hydrazide-bead conjugation, washing, and hydrolysis (Figure 4, top spectrum) even though peptides were present in the original mixture (Figure 4, bottom spectrum). Glycans such as DP5, DP6, and DP7 were recovered in the hydrolysis solution (top spectrum). The results demonstrated the ability of bead-hydrazide for glycan isolation and analysis.

The hydrazide-beads were then applied for the analysis of a serum sample. Proteins from 20 μL of a control human serum (approximately ~1 mg of total proteins or 10 μg of total glycans) were digested by trypsin, and glycans were released by PNGase F.^{42–44} The glycan and peptide mixture was dissolved into 100 μL of HPLC water. The estimated total amount of glycans was about 0.5 μg for 5 μL of prepared glycan mixture. The sample was mixed with standard glycans and peptides and then conjugated and hydrolyzed from the hydrazide beads followed by MALDI-MS analysis (Figure 5, top). It was observed that the standard glycans, DP5, DP6, and DP7, have a strong signal after conjugation-hydrolysis from beads while the standard peptides, angiotensin I (AG) and neurotensin (NT), were not detected in the released products. Additional glycans from serum were also detected and are shown in Figure 5 and Table 2. No signal was detected in the initial sample mixture due to the high concentration of ions in the complex mixture after denaturing and trypsin and PNGase F digestion (Figure 5, bottom). These results clearly showed that glycans were able to be isolated from the mixture using hydrazide beads.

We applied the reversible hydrazone capture-release method to the analysis of N-linked glycans from a human serum sample. A total of 17 glycans were detected by mass spectrometry after the capture-release process using this method as well four standard glycans that were spiked into human serum before glycans were isolated from the serum (Table 2). We expected that additional glycans were present in human serum samples, especially the complex glycans with terminal sialylic acids. The derivatization of sialylic acids by permethylation or esterification will facilitate the identification of sialylated glycans in mass spectrometry. In addition, releasing glycans from hydrazide beads using less acidic conditions will protect the hydrolysis of sialylated glycans. Finally, an increased amount of serum samples used for glycan isolation and separation of glycans from complex samples using liquid chromatography before mass spectrometry analysis can be used to identify low-abundance glycans.

CONCLUSIONS

As a highly specific and novel method, reversible hydrazone solid-phase extraction (rHSPE) is developed for glycan isolation from proteins, peptides, and other contaminants for glycan analysis. After glycans are released from glycoproteins, glycans in complex mixture were directly conjugated onto hydrazide-beads via reversible hydrazone by reducing ends of the glycans from the mixture. The hydrazide-glycan conjugation is chemically specific,

providing the unique means for the removal of other nonglycan molecules in the complex sample before the glycans are hydrolyzed and recovered for MS analysis. The hydrazone formation and hydrolysis allows for the analysis of glycans in its intact structure. The hydrazide coated on solid-phase surfaces such as ones on the slide are shown to be useful for on surface glycan capture and on target glycan analysis. The recovery of glycans was optimized in this study using the standard glycan and peptide mixture and could be improved further by optimizing reaction conditions such as irradiation energy, buffer pH, and temperature.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

- (1). Dennis JW, Granovsky M, Warren CE. *Biochim. Biophys. Acta, Gen. Subj.* 1999; 1473:21–34.
- (2). Reiter RE, Gu ZN, Watabe T, Thomas G, Szigeti K, Davis E, Wahl M, Nisitani S, Yamashiro J, Le Beau MM, Loda M, Witte ON. *Proc. Natl. Acad. Sci. U.S.A.* 1998; 95:1735–1740. [PubMed: 9465086]
- (3). Bertozzi CR, Dube DH. *Nat. Rev. Drug Discovery.* 2005; 4:477–488. [PubMed: 15931257]
- (4). de Boer RA, Voors AA, Muntendam P, van Gilst WH, van Veldhuisen DJ. *Eur. J. Heart Fail.* 2009; 11:811–817. [PubMed: 19648160]
- (5). Seidman JG, Seidman C. *Cell.* 2001; 104:557–567. [PubMed: 11239412]
- (6). Jaeken J, Matthijs G. *Annu. Rev. Genomics Hum. Genet.* 2007; 8:261–278. [PubMed: 17506657]
- (7). Rudd PM, Butler M, Quelhas D, Critchley AJ, Carchon H, Hebestreit HF, Hibbert RG, Vilarinho L, Teles E, Matthijs G, Schollen E, Argibay P, Harvey DJ, Dwek RA, Jaeken J. *Glycobiology.* 2003; 13:601–622. [PubMed: 12773475]
- (8). Jefferis R. *Nat. Rev. Drug Discovery.* 2009; 8:226–234. [PubMed: 19247305]
- (9). Walsh G, Jefferis R. *Nat. Biotechnol.* 2006; 24:1241–1252. [PubMed: 17033665]
- (10). Dove A. *Nat. Biotechnol.* 2001; 19:913–917. [PubMed: 11581651]
- (11). Dennis JW, Nabi IR, Demetriou M. *Cell.* 2009; 139:1229–1241. [PubMed: 20064370]
- (12). Esko JD, Fuster MM. *Nat. Rev. Cancer.* 2005; 5:526–542. [PubMed: 16069816]
- (13). Zhang H, Li XJ, Martin DB, Aebersold R. *Nat. Biotechnol.* 2003; 21:660–666. [PubMed: 12754519]
- (14). Larsen K, Thygesen MB, Guillaumie F, Willats WGT, Jensen KJ. *Carbohydr. Res.* 2006; 341:1209–1234. [PubMed: 16716275]
- (15). Kaji H, Saito H, Yamauchi Y, Shinkawa T, Taoka M, Hirabayashi J, Kasai K, Takahashi N, Isobe T. *Nat. Biotechnol.* 2003; 21:667–672. [PubMed: 12754521]
- (16). Tian YA, Zhou Y, Elliott S, Aebersold R, Zhang H. *Nat. Protoc.* 2007; 2:334–339. [PubMed: 17406594]
- (17). Li Y, Sokoll LJ, Rush J, Meany D, Zou N, Chan DW, Zhang H. *Proteomics: Clin. Appl.* 2009; 3:597–608.
- (18). Sun SS, Yang GL, Wang T, Wang QZ, Chen C, Li Z. *Anal. Bioanal. Chem.* 2010; 396:3071–3078. [PubMed: 20169334]

- (19). Zhang H, Loriaux P, Eng J, Campbell D, Keller A, Moss P, Bonneau R, Zhang N, Zhou Y, Wollscheid B, Cooke K, Yi EC, Lee H, Peskind ER, Zhang J, Smith RD; Aebersold R. *Genome Biol.* 2006; 7:R73. [PubMed: 16901351]
- (20). Li Y, Tian YA, Rezai T, Prakash A, Lopez MF, Chan DW, Zhang H. *Anal. Chem.* 2011; 83:240–245. [PubMed: 21141837]
- (21). Tian YA, Kelly-Spratt KS, Kemp CJ, Zhang HJ. *Proteome Res.* 2010; 9:5837–5847.
- (22). Tarentino AL, Gomez CM, Plummer TH. *Biochemistry.* 1985; 24:4665–4671. [PubMed: 4063349]
- (23). Regnier F, Geng M, Zhang X, Bina M. J. *Chromatogr., B.* 2001; 752:293–306.
- (24). Van den Steen P, Rudd PM, Dwek RA, Opdenakker G. *Crit. Rev. Biochem. Mol. Biol.* 1998; 33:151–208. [PubMed: 9673446]
- (25). Helenius A, Aeby M. *Science.* 2001; 291:2364–2369. [PubMed: 11269317]
- (26). Bynum MA, Yin HF, Felts K, Lee YM, Monell CR, Killeen K. *Anal. Chem.* 2009; 81:8818–8825. [PubMed: 19807107]
- (27). Rudd PM, Dwek RA. *Curr. Opin. Biotechnol.* 1997; 8:488–497. [PubMed: 9265730]
- (28). Harz H, Burgdorf K, Holtje JV. *Anal. Biochem.* 1990; 190:120–128. [PubMed: 2285138]
- (29). Neususs C, Balaguer E. *Anal. Chem.* 2006; 78:5384–5393. [PubMed: 16878873]
- (30). Gilar M, Ahn J, Bones J, Yu YQ, Rudd PM. *J. Chromatogr., B.* 2010; 878:403–408.
- (31). Deguchi K, Takegawa Y, Ito H, Keira T, Nakagawa H, Nishimura SI. *J. Sep. Sci.* 2006; 29:2533–2540. [PubMed: 17154134]
- (32). Neususs C, Balaguer E, Demelbauer U, Pelzing M, Sanz-Nebot V, Barbosa J. *Electrophoresis.* 2006; 27:2638–2650. [PubMed: 16817164]
- (33). Winchester B, Mills P, Mills K, Clayton P, Johnson A, Whitehouse D. *Biochem. J.* 2001; 359:249–254. [PubMed: 11583570]
- (34). Mills PB, Mills K, Mian N, Winchester BG, Clayton PT. *J. Inherit. Metab. Dis.* 2003; 26:119–134. [PubMed: 12889655]
- (35). Packer NH, Lawson MA, Jardine DR, Redmond JW. *Glycoconjugate J.* 1998; 15:737–747.
- (36). Larsen MR, Hojrup P, Roepstorff P. *Mol. Cell. Proteomics.* 2005; 4:107–119. [PubMed: 15561728]
- (37). Zou Z, Ibisate M, Zhou Y, Aebersold R, Xia YN, Zhang H. *Anal. Chem.* 2008; 80:1228–1234. [PubMed: 18197692]
- (38). Bhat VT, Caniard AM, Luksch T, Brenk R, Campopiano DJ, Greaney MF. *Nat. Chem.* 2010; 2:490–497. [PubMed: 20489719]
- (39). Dirksen A, Dirksen S, Hackeng TM, Dawson PE. *J. Am. Chem. Soc.* 2006; 128:15602–15603. [PubMed: 17147365]
- (40). Walker SH, Papas BN, Comins DL, Muddiman DC. *Anal. Chem.* 2010; 82:6636–6642. [PubMed: 20590124]
- (41). Roe MR, Xie HW, Bandhakavi S, Griffin TJ. *Anal. Chem.* 2007; 79:3747–3756. [PubMed: 17437329]
- (42). Spiro RG. *J. Biol. Chem.* 1960; 235:2860–2869. [PubMed: 16479679]
- (43). Spiro RG. *J. Biol. Chem.* 1962; 237:382–388. [PubMed: 13915839]
- (44). Kita Y, Miura Y, Furukawa J, Nakano M, Shinohara Y, Ohno M, Takimoto A, Nishimura S. *Mol. Cell. Proteomics.* 2007; 6:1437–1445. [PubMed: 17522412]

Figure 1. Schematic diagram of glycan capture by reversible hydrazone solid-phase extraction (rHSPE) for glycan analysis. Glycans are first conjugated; nonglycan molecules are removed by washing, and glycans are hydrolyzed from the solid phase in acidic buffer.

Figure 2.

Schematic diagram of capture and release glycans using hydrazide on solid-phase beads. Aldehyde is first formed from cyclic form under catalysis of aniline. At an acidic condition (pH 3–5), the hydrazide on beads conjugates to glycan by microwave irradiation and formation of hydrazone between glycan and the bead. The uncoupled components in the sample mixture are washed away from the beads. At a strong acidic condition (pH 1–2), the hydrazone is hydrolyzed to release glycan from the bead.

Figure 3.

Reversible hydrazone formation of Man-9 to ADH. The conjugation was in 10 mM acetate buffer (pH 5.0) and 10 mM aniline using microwave radiation. The molecular weight of Man-9/ADH conjugation is 2062.63 Da (mono- Na^+) (middle spectrum). Without conjugation prior to reaction, the mass spectrum (bottom) only showed Man-9 (mono- Na^+) (1906.68 Da); after reaction with ADH, the mass was 2062.63 Da (mono- Na^+) (middle); after hydrolysis by heating at 60 °C/1 h in 10% formic acid, Man-9 was recovered, indicating hydrolysis of hydrazone.

Figure 4.

Glycan conjugation and hydrolysis on hydrazide beads from a mixture of glycans (DPs) and peptides (NT, neurotensin and AG, angiotensin I). The mixed sample includes DP5 (851.25 Da), DP6 (1013.28 Da), DP7 (1175.31 Da), angiotensin I (mono- H^+) (1296.68 Da), and neurotensin (mono- H^+) (1672.77 Da) (peptide peaks other than angiotensin I and neurotensin were detected in mixed samples, which were due to the impurity of sample purchased). After conjugation, washing, and hydrolysis, glycans (DP5, DP6, and DP7) were detected by MALDI-MS. Angiotensin I and neurotensin were detected in washing solution but not in solution hydrolyzed from the bead surface.

Figure 5. Glycan isolation from human serum with the addition of standard glycans and peptides by hydrazide beads. The bottom spectrum was the sample mixture without glycan isolation. The top spectrum was isolated glycans from hydrazide beads after conjugation, washing, and hydrolysis.

Table 1
Glycan and Peptide Mixtures Used for Hydrazide-Bead Conjugation and Hydrolysis^a

samples	concn (mM)	vol (μ L)	peptides	concn (mM)	vol (μ L)	methanol/ acetic acid (μ L)	total (μ L)
DPs	10	10	NT-AG	0.1	0	10	20
DPs	10	0	NT-AG	0.1	2	18	20
DPs	10	10	NT-AG	0.1	2	8	20
DPs serum	10	$\frac{10}{5}$	NT-AG	0.1	2	3	20

^aDPs consisted of a 10 mM mixture of DP5, DP6, and DP7. A volume of 5 μ L of serum solution was used followed by digestion of trypsin and PNGase F. NT, neurotensin; AG, angiotensin I.

Table 2
Glycans Harvested from Human Sera Using Hydrazide Chemistry on Solid-Phase
Capture-Release^a

Mass (Da)	Glycan structure	SN	
		Average	CV
689.28		249	31.05%
851.25		135	29.42%
933.3		28	21.91%
1013.26		788	30.58%
1095.32		30	39.61%
1175.28		804	20.48%
1257.3		12	40.48%
1298.34		20	19.86%
1339.48		5	26.49%
1419.64		6	21.88%
1444.43		13	6.28%
1460.34		26	9.52%
1485.62		5	28.69%
1501.46		9	23.97%
1622.44		6	31.49%
1647.45		12	18.00%
1663.39		27	28.01%

Mass (Da)	Glycan structure	SN	
		Average	CV
1688.55		5	28.69%
1809.39		7	28.44%
1850.43		4	28.57%
1866.44		5	28.69%

^aThe control samples, DP4, DP5, DP6, and DP7, were given in the table for comparison.