glycoproteomics, is far from sufficient to identify glycosylation biomarkers for early cancer diagnosis. Here we discuss about issues and resolutions in the use of lectins by introducing methods for sample preparation prior to lectin chromatography. In addition to lectin-based approaches which have been most frequently utilized in glycomics, other sophisticated glyco-capturing technologies are reviewed. Because the concept required for biomarker discovery phase or preclinical validation phase is fundamentally different, the adequate methodologies for each are separately described.

2 Glycoproteomics for biomarker screening

2.1 Lectins or chemicals

Nowadays, state-of-the-art glycoproteomic technologies have been developed to enrich glycoproteins or glycopeptides from crude serum samples. These technologies are separated into lectin-based methods and chemical-based methods in principle. Which is better for the purpose of carbohydrate-targeting tumor marker discovery? If we intend to identify glycan structure changes as tumor markers, rather than concentration of core proteins, lectin is the only enrichment tool recognizing specific oligosaccharide linkages, excepting sialic acid specific chemistry Reverse Glycoblotting [35, 36]. The chemical enrichment of glycopeptides, such as using hydrazide chemistry [37-42], boronic acid [43-45], or hydrophilic interactions [46-48], certainly exhibits rigid interaction with glycan moieties, whereas most of lectin-glycan interactions are fragile [49]. However all of the chemical approaches above are based on covalent or affinity bond with rich hydroxyl groups on oligosaccharides, resulting in comprehensive and structure-unspecific capture of glycopeptides. Therefore the chemical route is inadvisable for glycan structure-targeting biomarker

Accepted Article

This article is protected by copyright. All rights reserved.

discovery. From the view of such features, we would like to focus on lectin-based glycopeptide enrichment methods in the following sections.

2.2 Using lectin column chromatography for glycoproteomics

Assuming that the eluate of lectin column chromatography would be analyzed in LC/MS/MS, it must be a critical issue whether we load proteins or digested peptides to lectin columns. When undigested serum proteins are purified with lectin column chromatography and eluted by hapten sugars, targeted glycoproteins would be eluted with a lot of nonspecific proteins and high concentration of hapten sugars, which could not be appropriate for mass spectrometric analysis (**Fig. 1 A**). The large amount of non-glycosylated protein elution is mainly caused by the limitation of solvent used in lectin column chromatography, with which the use of detergents, high salts, and organic solvents are not compatible. Furthermore, since most of serum proteins form complexes, co-elution of intact binding proteins is inevitable. More importantly, if enriched glycoproteins would have multiple glycosylation sites, it is hardly distinguishable which glycosylation sites might be associated with state of cancer.

On the other hand, when digested peptides are loaded to lectin columns, the purification efficiency of glycopeptides is relatively high due to the elimination of protein-protein interaction effect in the samples (**Fig. 1 B**). However elution of glycopeptides using hapten sugars still results in significant contamination of 200 – 1000 mM sugars in the eluate, which is not adequate for direct injection to LC/MS/MS. In addition to this fact, mass spectrometric analysis of eluted glycopeptides is also inefficient for comprehensive studies because automated

Accepted Article

This article is protected by copyright. All rights reserved.

Accepted Article

protein identification by database search is impossible for glycopeptides, for which additional deglycosylation steps would be finally required.

These characteristics of lectins on glycoproteomics have led to recent development of glycopeptidase elution technologies [46, 50]. After binding glycopeptides on lectin columns, PNGase-F elution in volatile salt buffers allows highly specific elution of only originally-glycosylated peptides. After lyophilizing eluate, the final product consists of completely deglycosylated peptides with no contaminant salts, which could be directly injected into LC/MS instruments and subjected to usual database search analysis on Mascot or Sequest software. Using this type of enzymatic elution procedure on click maltose HILIC beads, Zhu *et al.* successfully identified 92, 178, and 221 unique N-glycosylation sites from 10 nL, 100 nL, and 1 µL of human serum, respectively [46]. They effectively excluded desalting, buffer exchanging, and lyophilization steps to finish all pre-analytical procedures by spin columns within 1.5 hours.

Regarding peptide sequencing of enzymatically deglycosylated peptides on database search analysis, N-glycosylation sites are recognized as aspartic acid residues converted from asparagine residues by PNGase-F (**Fig. 1 C**). However chemically identical conversion may artificially occur on asparagine residues known as deamidation of asparagine. To eliminate the false positive identification of N-glycosylation sites by deamidation, we can utilize the PNGase-F reaction in heavy water ($H_2{}^{18}O$) [50-57]. The incorporation of ^{18}O into glycosylated asparagine residues by PNGase-F induces generation of 3 Da-increased asparagine residues, providing N-glycosylation site-specific stable isotope tags on peptides (**Fig. 1C**). Our team recently integrated on-column PNGase-F elution with the ^{18}O stable isotope labeling method and reported as an effective glycoproteomic biomarker screening technology,

This article is protected by copyright. All rights reserved.

Accepted Article

named isotopic glycosidase elution and labeling on lectin-column chromatography (IGEL) [50].

Hence issues on biochemical properties of lectins for glycoproteomics can be overcome by employing both protease digestion before lectin column purification and on-column glycopeptidase elution of peptides.

2.3 Pre-enrichment of glycopeptides prior to lectin purification

Lectin columns are convenient and widely-used enrichment tools for glycoproteins or glycopeptides. As contrasted with the benefits, enrichment ratio itself is not necessarily high enough. When we purified tryptic digest of crude serum by various types of 9 different lectin columns according to IGEL method described above, the enrichment efficiency (number of glycopeptide identification / total peptide identification × 100) was only 20 - 45%. Such insufficient enrichment efficiency was mainly caused by weak lectin-oligosaccharide affinities and the fact that abundance of non-glycosylated peptides in tryptic digest of crude serum samples was absolutely higher than that of glycosylated peptides. This aspect emphasizes the importance of adequate pre-enrichment techniques for pan-glycosylated peptides, such as cellulose column [58-60], graphite carbon column [61-65], and other hydrophilic affinity resins. Sergei *et al.* constructed C_{18}-cellulose mix mode column chromatography and achieved high-yield extraction of N- and O-glycosylated peptides from mixture of 10 standard proteins [59]. Lam *et al.* applied an online combination of reversed-phase/reversed-phase (RP-RP) and porous graphite carbon (PGC) liquid chromatography to the comprehensive analysis of ConA lectin-purified human serum samples and identified 134 N-glycosylated serum proteins, 151 possible N-glycosylation sites, and more than 40 possible N-glycan structures [65].

This article is protected by copyright. All rights reserved.

Accepted Article

The CL-4B Sepharose-based hydrophilic extraction of glycopeptides is also one of the most popular techniques in glycoproteomics. Selman et al. loaded 5 µl of CL-4B Sepharose beads into 96-well format plate and enriched human IgG-derived glycopeptides for matrix-assisted laser desorption ionization Fourier transform ion cyclotron resonance mass spectrometry (MALDI-FTICR-MS) [66]. This type of multiplexed purification system is especially suitable for large scale biomarker screening assays. Actually we also applied CL-4B Sepharose beads pre-enrichment procedure before lectin column purification and obtained finally around 90% glycopeptide enrichment ratio [50]. Thus pre-enrichment of total glycopeptides from complex peptide mixtures can drastically improve the glycopeptide focusing efficiency by subsequent lectin column chromatography.

2.4 Selection of lectins for glycoproteomics

So far hundreds of lectins have been isolated from plants, microbes, or animals and most of them are commercially available. The selection of lectins is a critical step for precise and comprehensive profiling of cancerous glycan disorders. In the glycoproteomic studies, both high specificity for glycan structures and high affinity to capture glycopeptides are needed. The glycopeptide enrichment ratio acquired from IGEL purification experiments using human serum and 9 distinct lectins (LCA, SNA-I, SNA-II, UEA-I, WGA, LPA, ConA, and SSA) was shown in **Table 1**. Concerning specificity of lectins, LCA, SNA-I, ConA and SSA demonstrated over 80% glycopeptide enrichment rate, suggesting that ligand specificity of these 4 lectins would be sufficient. Meanwhile when looking into numbers of glycopeptide identification which indirectly reflected the lectin-glycan affinity of each lectin, LCA and SNA-I lectins showed much less glycopeptide recovery rate compared to ConA

This article is protected by copyright. All rights reserved.

Accepted Article

or SSA. Additionally the remaining 5 lectins (Lotus, SNA-II, UEA-I, WGA, and LPA) were scarcely able to capture peptides. At least in our binding condition [100 mM ammonium bicarbonate, 5% acetonitrile, 1 mM calcium chloride, 1 mM manganese chloride], ConA and SSA lectin columns could be considered as appropriate materials to be used for specific and comprehensive profiling of human serum glycoproteome. Individual optimization would be required when analyzing other biological samples or using different condition of binding buffers. In order to cover a larger number of glycan structure changes, it is fundamental to increase options of lectins along with optimum purification protocols.

In recent studies, multi-lectin affinity chromatography (M-LAC) approaches were developed to enhance glycopeptide recovery rate and expand the comprehensiveness of targeted glycan structures. Zeng *et al.* combined high abundance protein depletion, ConA-Jacalin-WGA M-LAC, IEF separation, and LC-MS analysis and identified breast cancer associated proteins such as thrombospondin-1 and 5, alpha-1B-glycoprotein, serum amyloid P-component, and tenascin-X, which had potentially abnormal glycans [67]. The same group rigorously evaluated the identical lectin mixture in several glycoproteome profiling studies [68-72]. Qiu et al. integrated serial lectin purification by ConA and SNA with d0- or d3-*N*-acetoxysuccinamide stable isotope labeling on α-amino groups. By use of this methodology, they enabled effective enrichment of sialylated glycopeptides and also differential analysis of those [73].

2.5 Quantitative assessment of glycan structure changes

To identify cancer-associated alterations of glycosylation on multiple proteins, establishment of rigorous quantification strategies should be essential, which could

This article is protected by copyright. All rights reserved.

Accepted Article

stoichiometrically evaluate the changing rate of each glycoform. Comparative quantification results of only enriched glycopeptides are affected by not only glycan structure changes but also concentration of original core proteins itself, indicating that it is hard to determine whether the identified candidates might be glycosylation-targeting biomarkers or protein concentration biomarkers. Therefore subtraction of protein concentration effects from quantification results of lectin-enriched glycopeptides is necessary (**Fig. 2**). Recently we demonstrated a practical example of this concept for the identification of carbohydrate-targeting lung cancer biomarker discovery [50]. Here we acquired relative quantification profiles from both lectin-purified glycopeptides and pre-enriched non-glycosylated peptides by LC/MS/MS analysis individually. Finally the site-specific glycoform changes were determined by subtracting core protein concentrations calculated by non-glycosylated peptides from quantification results of each glycopeptide. This approach can illuminate the glycan structure changes on diverse glycosylation sites individually.

2.6 Quantitative glycoproteomic approaches for O-glycans

Although tools for comprehensive analysis of O-glycosylation are still limited compared with the N-glycomics, recent development of sophisticated chemistries have potential to be breakthrough technologies for O-glycan biomarker discovery. Hang *et al.* developed a metabolic labeling approach which utilized incorporation of tetra-aceylated-N-azidoacetylgalactosamine (GalNAz) into the reducing terminus of O-glycosylation sites [74, 75]. Following cell culture in the presence of GalNAz for several days, O-glycosylated proteins can be specifically collected by alkyne-activated resins (click chemistry) (**Fig. 3**). Using this technology, Slade *et al.* identified 267 potentially O-glycosylated proteins from the secretome of CHO cells

This article is protected by copyright. All rights reserved.

Accepted Article

[76]. Furthermore the Bertozzi's group succeeded to apply GalNAz chemistry to rapid profiling of O-linked glycoproteins in living mice [77] and also *in vivo* imaging of membrane-associated glycans in zebrafish [78]. The other group expanded this technology by using GalNAz, ManNAz, and GlcNAz to discover cell surface differentiation markers on human mesenchymal stem cells (hMSCs) [79]. Integration of such metabolic labeling methods for O-glycans with mass spectrometric structural analysis may facilitate comprehensive screening of fine O-glycan structure alterations in the future.

3 Glycoproteomics for High-throughput Biomarker Validation

For clinical application of biomarkers, pre-clinical validation experiments are usually required using independent larger sample set and high throughput quantification methods. In general, most of protein biomarkers are quantitatively measured by immunoassays in the validation phase, which include sandwich ELISA, AlphaLISA [80-84], and Luminex technology [85-89]. However it is often extremely difficult to make specific antibodies against both detailed glycan structures and glycosylation sites mainly because of the low immunogenicity of oligosaccharides and structural hindrance of amino acid epitopes by glycans. Thus alternative glycoproteomic technologies must be established for the replication assays using hundreds of clinical samples, which could detect site-specific glycan structure changes quantitatively with high throughput manner from complex protein mixtures, such as crude serum/plasma. Here we introduce a couple of technologies used in the glycosylation biomarker validation area.

This article is protected by copyright. All rights reserved.

Accepted Article

3.1 Lectin-antibody sandwich ELISA

A sandwich-type enzyme-linked immunosorbent assay (ELISA) using lectin and analyte specific antibody was originally developed by Drouin *et al.* in 1988 [90]. They intended to establish high throughput diagnostic assay for Bernard Soulier syndrome (BSS) which was a rare autosomal recessive coagulopathy leading to a deficiency of glycoprotein Ib (GpIb), an important clotting regulator as the receptor for von Willebrand factor. In that study they immobilized wheat germ agglutinin (WGA) lectin on 96 well plates to capture GpIb in prepared human platelet proteins and eventually succeeded to detect GpIb by specific monoclonal antibody AN51 quantitatively. They mentioned that this approach was simple, inexpensive and sensitive way to quantitate glycoproteins for which specific lectins and monoclonal antibodies were available. Tojo *et al.* applied the same concept of assay utilizing ConA-immobilized ELISA plates and specific polyclonal antibody to quantitate D-mannans of *Candida albicans* [91]. Since ConA lectin possessed a high binding specificity for the D-mannopyranose unit, the sensitivity and specificity of D-mannan detection were significantly improved compared to previous antibody-antibody ELISA or quantitative precipitin reaction (QPR), they reported.

In cancer diagnostic studies, Parker reported the application of lectin/antibody sandwich ELISA assay to the serological diagnosis of pancreatic cancer [92]. His team captured glycoproteins possessing *N*-acetylglucosamie and sialic acid moieties by WGA lectin and detected by CAM17.1 monoclonal antibody specific to a part of mucins. They provided diagnostic assay results from not only retrospective study showing a sensitivity of 78% for pancreatic cancer with a specificity of 76% [93], but also prospective study showing even better sensitivity and specificities (84 and 92%, respectively), suggesting that the assay probably performed better on fresh samples.

This article is protected by copyright. All rights reserved.

Accepted Article

They also circumstantially summarized the usefulness of CAM 17.1/WGA test for pancreatic cancer diagnosis in Lancet journal [94]. Very recently Miyoshi's group found that fucosylated haptoglobin had a great potential for the detection of pancreatic cancer and prognosis of postoperative colorectal cancer [95, 96]. They coated 96 well plates with anti-haptoglobin Fab antibody because IgG had fucosylated oligosaccharides in its Fc portion, and detected fucosylated haptoglobin by biotinylated AAL lectin [95-98]. They measured serum samples from 397 individuals and concluded that the sensitivity and specificity for the diagnosis of pancreatic cancer patients from normal controls was 50% and 91%, respectively [98].

3.2 Energy resolved oxonium ion monitoring (Erexim) technology

For the purpose of first high-throughput site-specific quantification of glycan structure variations, we recently developed energy resolved oxonium ion monitoring (Erexim) technology [99]. The oxonium ions are defined as any oxygen cations with three bonds in chemistry, while they are used synonymously with sugar oxonium ions in glycoproteomics, which are produced as oligosaccharide fragment ions in collision cells of mass spectrometers during collision induced dissociation (CID) of glycopeptides [100]. Typical oxonium ions (m/z) and the corresponding oligosaccharide components are shown in the right side of **Fig. 4**. The detection of particular set of oxonium ions in MS/MS spectra of glycopeptides not only represents the existence of glycan modifications but also provides signature of original glycan structures [101]. Furthermore we found that monitoring the yields of oxonium ions over a wide range of collision energy by use of multiple reaction monitoring (MRM [102-104]) on quadrupole mass spectrometer exhibited a highly glycan structure-unique fragmentation patterns. Indeed the Erexim curves allowed us to clearly

This article is protected by copyright. All rights reserved.

Accepted Article

distinguish even structural isomers (same mass with distinct glycan linkage) and separately quantify their contents (**Fig. 4**). This technology was then applied to the N-glycan profiling of three model therapeutic antibody drugs, Herceptin (trastuzumab), Avastin (bevacizumab), and Erbitux (cetuximab). In the lot-to-lot glycan structure variation test for Herceptin or Avastin, around 30 glycan structures on Fc region of antibody drugs were relatively quantified in 30 minutes analysis, revealing that at most 10% increase or decrease of several glycoforms were observed in both drugs (n = 4 for each of 4 lots). In the case of Avastin glycan profiling, glycans on both Fab and Fc regions were simultaneously quantified in a single Erexim analysis. The result showed that most of glycans on Fab region were non-human type glycan structures possessing N-glycolylneuraminic acids (Neu5Gc) or Gal (α1-3) Gal structures, whereas Fc region had conserved non-immunogenic glycan structures. The limit of detection and dynamic range of this technology were 30 attomole and more than 4 orders, respectively. Since the required sample preparation prior to mass spectrometric analysis was only usual trypsin digestion, the Erexim procedure would be appropriate for automated high-throughput analysis. Thus this technology has enough potential to be applied to routine evaluation of drug quality, safety, and potency, for which extremely high reproducibility, quantitative capability, and throughput. Meanwhile our Erexim profiling technique is promising technology allowing rapid and site-specific validation of extracted glycan structure-targeted tumor marker candidates using multiple crude specimens.

4 Toward Industrialization and Approval of Carbohydrate-targeting Biomarkers

This article is protected by copyright. All rights reserved.

Accepted Article

Biomarkers developed for commercial use and regulatory approval must be required to present data supporting validity and clinical utility. According to the FDA Guidance for industry: Pharmacogenomic data submissions 2005, a valid biomarker is a biomarker that is measured in an analytical test system with "well-established performance characteristics" and for which there is an "established scientific framework or body of evidence that elucidates the clinical significance of test results." In case of carbohydrate-targeting biomarkers for cancer early detection or prognosis, the developed devices need to be approved as *in vitro* diagnostics.

Toward that purpose, a couple of key requirements have to be fulfilled. Firstly development of high-throughput, easy-to-use, reproducible, and not so expensive diagnostic devices would be required, which are suitable for widespread clinical use. As described in Section 3.1, lectin-antibody sandwich ELISA system could be the first choice satisfying all of these criteria. However, in some cases it would be difficult to achieve sensitive detection for low abundant serum glycoproteins because of insufficient specificity and affinity of lectins for the glycan epitopes. The Erexim technology (Section 3.2) can provide extremely high sensitivity, specificity, and reproducibility for the quantitative assessment of site-specific glycan structure disorders, whereas the high-spec mass spectrometer and proficient skills of LC/MS/MS operation are required.

The other requirement toward the approval of diagnostics is biological evidence explaining how and why the targeted glycan structure alterations would occur on a particular serum glycoprotein in cancer patients. It is often complicated to figure out the origin of biomarker glycoproteins and biochemical mechanisms of abnormal oligosaccharide generation, especially when investigating for glycoproteins identified from serum shotgun proteomics-driven biomarker screening. To present scientifically

This article is protected by copyright. All rights reserved.

Accepted Article

strong evidences for carbohydrate-targeting biomarker candidates, further basic and detailed glycobiological studies will be necessary, including analysis of cellular glycan biosynthesis pathways, comprehensive expression analysis of glycosyltransferases, or confirmation of glycoform changes in cancer tissues. Future development of more sensitive, high-throughput, and site-specific glycan structure profiling technologies are also fundamental to facilitate clinical application of numerous carbohydrate-targeting biomarker candidates reported previously on papers.

This article is protected by copyright. All rights reserved.

Accepted Article

References

[1] Anderson, N. L., The clinical plasma proteome: a survey of clinical assays for proteins in plasma and serum. *Clinical chemistry* 2010, *56*, 177-185.
[2] Anderson, N. L., Anderson, N. G., The human plasma proteome: history, character, and diagnostic prospects. *Molecular & cellular proteomics : MCP* 2002, *1*, 845-867.
[3] Ueda, K., Saichi, N., Takami, S., Kang, D., *et al.*, A comprehensive peptidome profiling technology for the identification of early detection biomarkers for lung adenocarcinoma. *PloS one* 2011, *6*, e18567.
[4] Schulz-Knappe, P., Zucht, H. D., Heine, G., Jurgens, M., *et al.*, Peptidomics: the comprehensive analysis of peptides in complex biological mixtures. *Combinatorial chemistry & high throughput screening* 2001, *4*, 207-217.
[5] Machtejevas, E., Andrecht, S., Lubda, D., Unger, K. K., Monolithic silica columns of various format in automated sample clean-up/multidimensional liquid chromatography/mass spectrometry for peptidomics. *Journal of chromatography. A* 2007, *1144*, 97-101.
[6] Fogli, A., Bulet, P., Peptidomics analysis of lymphoblastoid cell lines. *Methods in molecular biology* 2010, *615*, 247-257.
[7] Overall, C. M., Dean, R. A., Degradomics: systems biology of the protease web. Pleiotropic roles of MMPs in cancer. *Cancer metastasis reviews* 2006, *25*, 69-75.
[8] Doucet, A., Butler, G. S., Rodriguez, D., Prudova, A., Overall, C. M., Metadegradomics: toward in vivo quantitative degradomics of proteolytic post-translational modifications of the cancer proteome. *Molecular & cellular proteomics : MCP* 2008, *7*, 1925-1951.
[9] Butler, G. S., Dean, R. A., Smith, D., Overall, C. M., Membrane protease degradomics: proteomic identification and quantification of cell surface protease substrates. *Methods in molecular biology* 2009, *528*, 159-176.
[10] Huesgen, P. F., Overall, C. M., N- and C-terminal degradomics: new approaches to reveal biological roles for plant proteases from substrate identification. *Physiologia plantarum* 2012, *145*, 5-17.
[11] Wejda, M., Impens, F., Takahashi, N., Van Damme, P., *et al.*, Degradomics reveals that cleavage specificity profiles of caspase-2 and effector caspases are alike. *The Journal of biological chemistry* 2012, *287*, 33983-33995.
[12] Wehr, A. Y., Hwang, W. T., Blair, I. A., Yu, K. H., Relative quantification of serum proteins from pancreatic ductal adenocarcinoma patients by stable isotope dilution liquid chromatography-mass spectrometry. *Journal of proteome research* 2012, *11*, 1749-1758.
[13] Shi, T., Zhou, J. Y., Gritsenko, M. A., Hossain, M., *et al.*, IgY14 and SuperMix immunoaffinity separations coupled with liquid chromatography-mass spectrometry for human plasma proteomics biomarker discovery. *Methods* 2012, *56*, 246-253.
[14] Koutroukides, T. A., Guest, P. C., Leweke, F. M., Bailey, D. M., *et al.*, Characterization of the human serum depletome by label-free shotgun proteomics. *Journal of separation science* 2011, *34*, 1621-1626.
[15] Beer, L. A., Tang, H. Y., Sriswasdi, S., Barnhart, K. T., Speicher, D. W., Systematic discovery of ectopic pregnancy serum biomarkers using 3-D protein profiling coupled with label-free quantitation. *Journal of proteome research* 2011, *10*, 1126-1138.
[16] Berven, F. S., Ahmad, R., Clauser, K. R., Carr, S. A., Optimizing performance of glycopeptide capture for plasma proteomics. *Journal of proteome research* 2010, *9*, 1706-1715.
[17] Jagtap, P., Bandhakavi, S., Higgins, L., McGowan, T., *et al.*, Workflow for analysis of high mass accuracy salivary data set using MaxQuant and ProteinPilot search algorithm. *Proteomics* 2012, *12*, 1726-1730.

This article is protected by copyright. All rights reserved.

Accepted Article

[18] Milan, E., Lazzari, C., Anand, S., Floriani, I., *et al.*, SAA1 is over-expressed in plasma of non small cell lung cancer patients with poor outcome after treatment with epidermal growth factor receptor tyrosine-kinase inhibitors. *Journal of proteomics* 2012, *76 Spec No.*, 91-101.
[19] Righetti, P. G., Boschetti, E., The ProteoMiner and the FortyNiners: searching for gold nuggets in the proteomic arena. *Mass spectrometry reviews* 2008, *27*, 596-608.
[20] Boschetti, E., Righetti, P. G., The ProteoMiner in the proteomic arena: a non-depleting tool for discovering low-abundance species. *Journal of proteomics* 2008, *71*, 255-264.
[21] Hartwig, S., Czibere, A., Kotzka, J., Passlack, W., *et al.*, Combinatorial hexapeptide ligand libraries (ProteoMiner): an innovative fractionation tool for differential quantitative clinical proteomics. *Archives of physiology and biochemistry* 2009, *115*, 155-160.
[22] Fonslow, B. R., Carvalho, P. C., Academia, K., Freeby, S., *et al.*, Improvements in proteomic metrics of low abundance proteins through proteome equalization using ProteoMiner prior to MudPIT. *Journal of proteome research* 2011, *10*, 3690-3700.
[23] Hekmat, O., He, S., Warren, R. A., Withers, S. G., A mechanism-based ICAT strategy for comparing relative expression and activity levels of glycosidases in biological systems. *Journal of proteome research* 2008, *7*, 3282-3292.
[24] Kang, X., Sun, L., Guo, K., Shu, H., *et al.*, Serum protein biomarkers screening in HCC patients with liver cirrhosis by ICAT-LC-MS/MS. *Journal of cancer research and clinical oncology* 2010, *136*, 1151-1159.
[25] Ramus, C., Gonzalez de Peredo, A., Dahout, C., Gallagher, M., Garin, J., An optimized strategy for ICAT quantification of membrane proteins. *Molecular & cellular proteomics : MCP* 2006, *5*, 68-78.
[26] Sethuraman, M., McComb, M. E., Huang, H., Huang, S., *et al.*, Isotope-coded affinity tag (ICAT) approach to redox proteomics: identification and quantitation of oxidant-sensitive cysteine thiols in complex protein mixtures. *Journal of proteome research* 2004, *3*, 1228-1233.
[27] Yan, W., Lee, H., Deutsch, E. W., Lazaro, C. A., *et al.*, A dataset of human liver proteins identified by protein profiling via isotope-coded affinity tag (ICAT) and tandem mass spectrometry. *Molecular & cellular proteomics : MCP* 2004, *3*, 1039-1041.
[28] Li, J., Steen, H., Gygi, S. P., Protein profiling with cleavable isotope-coded affinity tag (cICAT) reagents: the yeast salinity stress response. *Molecular & cellular proteomics : MCP* 2003, *2*, 1198-1204.
[29] Ueda, K., Katagiri, T., Shimada, T., Irie, S., *et al.*, Comparative profiling of serum glycoproteome by sequential purification of glycoproteins and 2-nitrobenzensulfenyl (NBS) stable isotope labeling: a new approach for the novel biomarker discovery for cancer. *Journal of proteome research* 2007, *6*, 3475-3483.
[30] Iida, T., Kuyama, H., Watanabe, M., Toda, C., *et al.*, Rapid and efficient MALDI-TOF MS peak detection of 2-nitrobenzenesulfenyl-labeled peptides using the combination of HPLC and an automatic spotting apparatus. *Journal of biomolecular techniques : JBT* 2006, *17*, 333-341.
[31] Ou, K., Kesuma, D., Ganesan, K., Yu, K., *et al.*, Quantitative profiling of drug-associated proteomic alterations by combined 2-nitrobenzenesulfenyl chloride (NBS) isotope labeling and 2DE/MS identification. *Journal of proteome research* 2006, *5*, 2194-2206.
[32] Matsuo, E., Toda, C., Watanabe, M., Ojima, N., *et al.*, Selective detection of 2-nitrobenzenesulfenyl-labeled peptides by matrix-assisted laser desorption/ionization-time of flight mass spectrometry using a novel matrix. *Proteomics* 2006, *6*, 2042-2049.
[33] Matsuo, E., Toda, C., Watanabe, M., Iida, T., *et al.*, Improved 2-nitrobenzenesulfenyl method: optimization of the protocol and improved enrichment for labeled peptides. *Rapid communications in mass spectrometry : RCM* 2006, *20*, 31-38.

This article is protected by copyright. All rights reserved.

Accepted Article

[34] Kuyama, H., Watanabe, M., Toda, C., Ando, E., *et al.*, An approach to quantitative proteome analysis by labeling tryptophan residues. *Rapid communications in mass spectrometry : RCM* 2003, *17*, 1642-1650.
[35] Kurogochi, M., Amano, M., Fumoto, M., Takimoto, A., *et al.*, Reverse glycoblotting allows rapid-enrichment glycoproteomics of biopharmaceuticals and disease-related biomarkers. *Angewandte Chemie* 2007, *46*, 8808-8813.
[36] Kurogochi, M., Matsushista, T., Amano, M., Furukawa, J., *et al.*, Sialic acid-focused quantitative mouse serum glycoproteomics by multiple reaction monitoring assay. *Molecular & cellular proteomics : MCP* 2010, *9*, 2354-2368.
[37] Ziegler, A., Cerciello, F., Bigosch, C., Bausch-Fluck, D., *et al.*, Proteomic surfaceome analysis of mesothelioma. *Lung cancer* 2012, *75*, 189-196.
[38] Gundry, R. L., Riordon, D. R., Tarasova, Y., Chuppa, S., *et al.*, A cell surfaceome map for immunophenotyping and sorting pluripotent stem cells. *Molecular & cellular proteomics : MCP* 2012, *11*, 303-316.
[39] Danzer, C., Eckhardt, K., Schmidt, A., Fankhauser, N., *et al.*, Comprehensive description of the N-glycoproteome of mouse pancreatic beta-cells and human islets. *Journal of proteome research* 2012, *11*, 1598-1608.
[40] Bausch-Fluck, D., Hofmann, A., Wollscheid, B., Cell surface capturing technologies for the surfaceome discovery of hepatocytes. *Methods in molecular biology* 2012, *909*, 1-16.
[41] Kovarova, H., Gadher, S. J., Wollscheid, B., Focus on stem cell proteomics. *Proteomics* 2011, *11*, 3943-3945.
[42] Wollscheid, B., Bausch-Fluck, D., Henderson, C., O'Brien, R., *et al.*, Mass-spectrometric identification and relative quantification of N-linked cell surface glycoproteins. *Nature biotechnology* 2009, *27*, 378-386.
[43] Liu, L., Zhang, Y., Zhang, L., Yan, G., *et al.*, Highly specific revelation of rat serum glycopeptidome by boronic acid-functionalized mesoporous silica. *Analytica chimica acta* 2012, *753*, 64-72.
[44] Xu, Y., Zhang, L., Lu, H., Yang, P., On-plate enrichment of glycopeptides by using boronic acid functionalized gold-coated Si wafer. *Proteomics* 2010, *10*, 1079-1086.
[45] Tang, J., Liu, Y., Qi, D., Yao, G., *et al.*, On-plate-selective enrichment of glycopeptides using boronic acid-modified gold nanoparticles for direct MALDI-QIT-TOF MS analysis. *Proteomics* 2009, *9*, 5046-5055.
[46] Zhu, J., Wang, F., Chen, R., Cheng, K., *et al.*, Centrifugation assisted microreactor enables facile integration of trypsin digestion, hydrophilic interaction chromatography enrichment, and on-column deglycosylation for rapid and sensitive N-glycoproteome analysis. *Analytical chemistry* 2012, *84*, 5146-5153.
[47] Xiong, Z., Zhao, L., Wang, F., Zhu, J., *et al.*, Synthesis of branched PEG brushes hybrid hydrophilic magnetic nanoparticles for the selective enrichment of N-linked glycopeptides. *Chemical communications* 2012, *48*, 8138-8140.
[48] Kuo, C. W., Wu, I. L., Hsiao, H. H., Khoo, K. H., Rapid glycopeptide enrichment and N-glycosylation site mapping strategies based on amine-functionalized magnetic nanoparticles. *Analytical and bioanalytical chemistry* 2012, *402*, 2765-2776.
[49] Dam, T. K., Gerken, T. A., Brewer, C. F., Thermodynamics of multivalent carbohydrate-lectin cross-linking interactions: importance of entropy in the bind and jump mechanism. *Biochemistry* 2009, *48*, 3822-3827.
[50] Ueda, K., Takami, S., Saichi, N., Daigo, Y., *et al.*, Development of serum glycoproteomic profiling technique; simultaneous identification of glycosylation sites and site-specific quantification of glycan structure changes. *Molecular & cellular proteomics : MCP* 2010, *9*, 1819-1828.

This article is protected by copyright. All rights reserved.

Accepted Article

[51] Kaji, H., Saito, H., Yamauchi, Y., Shinkawa, T., *et al.*, Lectin affinity capture, isotope-coded tagging and mass spectrometry to identify N-linked glycoproteins. *Nature biotechnology* 2003, *21*, 667-672.
[52] Kaji, H., Shikanai, T., Sasaki-Sawa, A., Wen, H., *et al.*, Large-scale identification of N-glycosylated proteins of mouse tissues and construction of a glycoprotein database, GlycoProtDB. *Journal of proteome research* 2012, *11*, 4553-4566.
[53] Liu, T. W., Kaji, H., Togayachi, A., Ito, H., *et al.*, A chemoenzymatic approach toward the identification of fucosylated glycoproteins and mapping of N-glycan sites. *Glycobiology* 2012, *22*, 630-637.
[54] Narimatsu, H., Sawaki, H., Kuno, A., Kaji, H., *et al.*, A strategy for discovery of cancer glyco-biomarkers in serum using newly developed technologies for glycoproteomics. *The FEBS journal* 2010, *277*, 95-105.
[55] Sugahara, D., Kaji, H., Sugihara, K., Asano, M., Narimatsu, H., Large-scale identification of target proteins of a glycosyltransferase isozyme by Lectin-IGOT-LC/MS, an LC/MS-based glycoproteomic approach. *Scientific reports* 2012, *2*, 680.
[56] Han, H., Stapels, M., Ying, W., Yu, Y., *et al.*, Comprehensive characterization of the N-glycosylation status of CD44s by use of multiple mass spectrometry-based techniques. *Analytical and bioanalytical chemistry* 2012, *404*, 373-388.
[57] Zhang, S., Liu, X., Kang, X., Sun, C., *et al.*, iTRAQ plus 18O: a new technique for target glycoprotein analysis. *Talanta* 2012, *91*, 122-127.
[58] Kubota, K., Sato, Y., Suzuki, Y., Goto-Inoue, N., *et al.*, Analysis of glycopeptides using lectin affinity chromatography with MALDI-TOF mass spectrometry. *Analytical chemistry* 2008, *80*, 3693-3698.
[59] Snovida, S. I., Bodnar, E. D., Viner, R., Saba, J., Perreault, H., A simple cellulose column procedure for selective enrichment of glycopeptides and characterization by nano LC coupled with electron-transfer and high-energy collisional-dissociation tandem mass spectrometry. *Carbohydrate research* 2010, *345*, 792-801.
[60] Wada, Y., Tajiri, M., Yoshida, S., Hydrophilic affinity isolation and MALDI multiple-stage tandem mass spectrometry of glycopeptides for glycoproteomics. *Analytical chemistry* 2004, *76*, 6560-6565.
[61] Davies, M., Smith, K. D., Harbin, A. M., Hounsell, E. F., High-performance liquid chromatography of oligosaccharide alditols and glycopeptides on a graphitized carbon column. *Journal of chromatography* 1992, *609*, 125-131.
[62] Davies, M. J., Smith, K. D., Carruthers, R. A., Chai, W., *et al.*, Use of a porous graphitised carbon column for the high-performance liquid chromatography of oligosaccharides, alditols and glycopeptides with subsequent mass spectrometry analysis. *Journal of chromatography* 1993, *646*, 317-326.
[63] Fan, J. Q., Kondo, A., Kato, I., Lee, Y. C., High-performance liquid chromatography of glycopeptides and oligosaccharides on graphitized carbon columns. *Analytical biochemistry* 1994, *219*, 224-229.
[64] Thaysen-Andersen, M., Mysling, S., Hojrup, P., Site-specific glycoprofiling of N-linked glycopeptides using MALDI-TOF MS: strong correlation between signal strength and glycoform quantities. *Analytical chemistry* 2009, *81*, 3933-3943.
[65] Lam, M. P., Lau, E., Siu, S. O., Ng, D. C., *et al.*, Online combination of reversed-phase/reversed-phase and porous graphitic carbon liquid chromatography for multicomponent separation of proteomics and glycoproteomics samples. *Electrophoresis* 2011, *32*, 2930-2940.
[66] Selman, M. H., McDonnell, L. A., Palmblad, M., Ruhaak, L. R., *et al.*, Immunoglobulin G glycopeptide profiling by matrix-assisted laser desorption ionization Fourier transform ion cyclotron resonance mass spectrometry. *Analytical chemistry* 2010, *82*, 1073-1081.

This article is protected by copyright. All rights reserved.

Accepted Article

[67] Zeng, Z., Hincapie, M., Pitteri, S. J., Hanash, S., *et al.*, A proteomics platform combining depletion, multi-lectin affinity chromatography (M-LAC), and isoelectric focusing to study the breast cancer proteome. *Analytical chemistry* 2011, *83*, 4845-4854.
[68] Yang, Z., Hancock, W. S., Approach to the comprehensive analysis of glycoproteins isolated from human serum using a multi-lectin affinity column. *Journal of chromatography. A* 2004, *1053*, 79-88.
[69] Yang, Z., Hancock, W. S., Monitoring glycosylation pattern changes of glycoproteins using multi-lectin affinity chromatography. *Journal of chromatography. A* 2005, *1070*, 57-64.
[70] Dayarathna, M. K., Hancock, W. S., Hincapie, M., A two step fractionation approach for plasma proteomics using immunodepletion of abundant proteins and multi-lectin affinity chromatography: Application to the analysis of obesity, diabetes, and hypertension diseases. *Journal of separation science* 2008, *31*, 1156-1166.
[71] Kullolli, M., Hancock, W. S., Hincapie, M., Preparation of a high-performance multi-lectin affinity chromatography (HP-M-LAC) adsorbent for the analysis of human plasma glycoproteins. *Journal of separation science* 2008, *31*, 2733-2739.
[72] Zheng, X., Wu, S. L., Hincapie, M., Hancock, W. S., Study of the human plasma proteome of rheumatoid arthritis. *Journal of chromatography. A* 2009, *1216*, 3538-3545.
[73] Qiu, R., Regnier, F. E., Use of multidimensional lectin affinity chromatography in differential glycoproteomics. *Analytical chemistry* 2005, *77*, 2802-2809.
[74] Hang, H. C., Yu, C., Kato, D. L., Bertozzi, C. R., A metabolic labeling approach toward proteomic analysis of mucin-type O-linked glycosylation. *Proceedings of the National Academy of Sciences of the United States of America* 2003, *100*, 14846-14851.
[75] Laughlin, S. T., Bertozzi, C. R., Metabolic labeling of glycans with azido sugars and subsequent glycan-profiling and visualization via Staudinger ligation. *Nature protocols* 2007, *2*, 2930-2944.
[76] Slade, P. G., Hajivandi, M., Bartel, C. M., Gorfien, S. F., Identifying the CHO Secretome using Mucin-type O-Linked Glycosylation and Click-chemistry. *Journal of proteome research* 2012.
[77] Dube, D. H., Prescher, J. A., Quang, C. N., Bertozzi, C. R., Probing mucin-type O-linked glycosylation in living animals. *Proceedings of the National Academy of Sciences of the United States of America* 2006, *103*, 4819-4824.
[78] Laughlin, S. T., Baskin, J. M., Amacher, S. L., Bertozzi, C. R., In vivo imaging of membrane-associated glycans in developing zebrafish. *Science* 2008, *320*, 664-667.
[79] Hart, C., Chase, L. G., Hajivandi, M., Agnew, B., Metabolic labeling and click chemistry detection of glycoprotein markers of mesenchymal stem cell differentiation. *Methods in molecular biology* 2011, *698*, 459-484.
[80] Foo, J. Y., Wan, Y., Kostner, K., Arivalagan, A., *et al.*, NT-ProBNP Levels in Saliva and Its Clinical Relevance to Heart Failure. *PloS one* 2012, *7*, e48452.
[81] Pfluger, M., Kapuscik, A., Lucas, R., Koppensteiner, A., *et al.*, A Combined Impedance and AlphaLISA-Based Approach to Identify Anti-inflammatory and Barrier-Protective Compounds in Human Endothelium. *Journal of biomolecular screening* 2012.
[82] Peters, C. D., Jespersen, B., Norregaard, R., AlphaLISA versus ELISA-based detection of interleukin 18 in healthy subjects and patients with end-stage renal disease. *Scandinavian journal of clinical and laboratory investigation* 2012, *72*, 583-592.
[83] Cosentino, G., AlphaLISA assays to improve the vaccine development process. *Developments in biologicals* 2012, *134*, 107-111.
[84] Waller, H., Chatterji, U., Gallay, P., Parkinson, T., Targett-Adams, P., The use of AlphaLISA technology to detect interaction between hepatitis C virus-encoded NS5A and cyclophilin A. *Journal of virological methods* 2010, *165*, 202-210.

This article is protected by copyright. All rights reserved.

Accepted Article

[85] Biancotto, A., Feng, X., Langweiler, M., Young, N. S., Philip McCoy, J., Effect of anticoagulants on multiplexed measurement of cytokine/chemokines in healthy subjects. *Cytokine* 2012, *60*, 438-446.
[86] Kim, Y. W., Bae, S. M., Lim, H., Kim, Y. J., Ahn, W. S., Development of multiplexed bead-based immunoassays for the detection of early stage ovarian cancer using a combination of serum biomarkers. *PloS one* 2012, *7*, e44960.
[87] Rinewalt, D., Shersher, D. D., Daly, S., Fhied, C., *et al.*, Development of a serum biomarker panel predicting recurrence in stage I non-small cell lung cancer patients. *The Journal of thoracic and cardiovascular surgery* 2012, *144*, 1344-1351.
[88] Tait, B. D., Hudson, F., Cantwell, L., Brewin, G., *et al.*, Review article: Luminex technology for HLA antibody detection in organ transplantation. *Nephrology* 2009, *14*, 247-254.
[89] Picascia, A., Infante, T., Napoli, C., Luminex and antibody detection in kidney transplantation. *Clinical and experimental nephrology* 2012, *16*, 373-381.
[90] Drouin, J., Izaguirre, C. A., Patenaude, P., Quantitation of cell membrane glycoproteins in pathological conditions using a lectin-bound enzyme-linked immunosorbent assay (ELISA). Application to human platelets in the Bernard-Soulier syndrome. *Journal of immunological methods* 1988, *110*, 217-223.
[91] Tojo, M., Shibata, N., Osanai, T., Mikami, T., *et al.*, Sandwich enzyme-linked immunosorbent assay of D-mannans of Candida albicans NIH A-207 and NIH B-792 strains using concanavalin A and polyclonal rabbit anti-C. albicans antisera. *Carbohydrate research* 1991, *213*, 325-330.
[92] Parker, N., Lectin/Antibody "sandwich" ELISA for quantification of circulating mucin as a diagnostic test for pancreatic cancer. *Methods in molecular medicine* 1998, *9*, 249-253.
[93] Parker, N., Makin, C. A., Ching, C. K., Eccleston, D., *et al.*, A new enzyme-linked lectin/mucin antibody sandwich assay (CAM 17.1/WGA) assessed in combination with CA 19-9 and peanut lectin binding assay for the diagnosis of pancreatic cancer. *Cancer* 1992, *70*, 1062-1068.
[94] Yiannakou, J. Y., Newland, P., Calder, F., Kingsnorth, A. N., Rhodes, J. M., Prospective study of CAM 17.1/WGA mucin assay for serological diagnosis of pancreatic cancer. *Lancet* 1997, *349*, 389-392.
[95] Takeda, Y., Shinzaki, S., Okudo, K., Moriwaki, K., *et al.*, Fucosylated haptoglobin is a novel type of cancer biomarker linked to the prognosis after an operation in colorectal cancer. *Cancer* 2012, *118*, 3036-3043.
[96] Okuyama, N., Ide, Y., Nakano, M., Nakagawa, T., *et al.*, Fucosylated haptoglobin is a novel marker for pancreatic cancer: a detailed analysis of the oligosaccharide structure and a possible mechanism for fucosylation. *International journal of cancer. Journal international du cancer* 2006, *118*, 2803-2808.
[97] Miyoshi, E., Shinzaki, S., Moriwaki, K., Matsumoto, H., Identification of fucosylated haptoglobin as a novel tumor marker for pancreatic cancer and its possible application for a clinical diagnostic test. *Methods in enzymology* 2010, *478*, 153-164.
[98] Matsumoto, H., Shinzaki, S., Narisada, M., Kawamoto, S., *et al.*, Clinical application of a lectin-antibody ELISA to measure fucosylated haptoglobin in sera of patients with pancreatic cancer. *Clinical chemistry and laboratory medicine : CCLM / FESCC* 2010, *48*, 505-512.
[99] Toyama, A., Nakagawa, H., Matsuda, K., Sato, T. A., *et al.*, Quantitative structural characterization of local N-glycan microheterogeneity in therapeutic antibodies by energy-resolved oxonium ion monitoring. *Analytical chemistry* 2012, *84*, 9655-9662.
[100] Medzihradszky, K. F., Characterization of site-specific N-glycosylation. *Methods in molecular biology* 2008, *446*, 293-316.

This article is protected by copyright. All rights reserved.

Accepted Article

[101] Scott, N. E., Parker, B. L., Connolly, A. M., Paulech, J., *et al.*, Simultaneous glycan-peptide characterization using hydrophilic interaction chromatography and parallel fragmentation by CID, higher energy collisional dissociation, and electron transfer dissociation MS applied to the N-linked glycoproteome of Campylobacter jejuni. *Molecular & cellular proteomics : MCP* 2011, *10*, M000031-MCP000201.
[102] Lemoine, J., Fortin, T., Salvador, A., Jaffuel, A., *et al.*, The current status of clinical proteomics and the use of MRM and MRM(3) for biomarker validation. *Expert review of molecular diagnostics* 2012, *12*, 333-342.
[103] Kitteringham, N. R., Jenkins, R. E., Lane, C. S., Elliott, V. L., Park, B. K., Multiple reaction monitoring for quantitative biomarker analysis in proteomics and metabolomics. *Journal of chromatography. B, Analytical technologies in the biomedical and life sciences* 2009, *877*, 1229-1239.
[104] Shi, T., Su, D., Liu, T., Tang, K., *et al.*, Advancing the sensitivity of selected reaction monitoring-based targeted quantitative proteomics. *Proteomics* 2012, *12*, 1074-1092.

Footnotes

Competing interests – I have no conflict of interest to declare.

This article is protected by copyright. All rights reserved.