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Review Article

Glycopeptide enrichment and separation for protein glycosylation analysis

Protein glycosylation plays key roles in many biological processes. In addition, alterations in protein glycosylation have been related to different diseases, as well as may affect the properties of recombinant proteins used as human therapeutics. For this reason, protein glycosylation analysis is of main interest in biomedical and biopharmaceutical research. Although recent advances in LC-MS analysis have made possible glycoprotein glycosylation site identification, characterization of glycoprotein glycan structures, as well as glycoprotein identification and quantification, protein glycosylation analysis in complex samples still remains a difficult task. This is due to low proportions of glycopeptides in comparison to peptides obtained after glycoprotein digestion, the suppression of the glycopeptide MS signals in the presence of peptides, and the high heterogeneity of glycopeptides. Thus, in the recent years, continuous efforts have been devoted to the development of glycopeptide enrichment and separation strategies to facilitate and improve glycoprotein glycosylation analysis in complex samples. This review summarizes the different methodologies that can be employed for glycopeptide enrichment/separation from complex samples including methods based on lectin affinity enrichment, covalent interactions, or chromatographic separations and solid-phase extraction.

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1 Introduction

Protein glycosylation plays an important role in many cellular processes such as cell adhesion, receptor activation, and signal transduction. Protein glycosylation can significantly alter protein conformation and consequently activity and protein–protein interactions [1–3]. Altered glycosylation has been associated to different diseases such as cancer [4–6], neurodegenerative diseases [7], or rheumatoid arthritis [8], however, very little is known about the complex interrelations so far. In addition, glycoproteins are commonly employed as pharmaceuticals, and differences in glycosylation can affect bioactivity, pharmacokinetics, stability, immunogenicity, and allergenicity [9–11]. For these reasons, the analysis of protein glycosylation is very important to understand biological processes, to correlate changes in glycosylation with healthy and diseased states, as well as to assure the consistent quality of biopharmaceuticals. The most commonly employed technique for the analysis of protein glycosylation (glycosylation site identification, glycan structure determination, site occupancy, and glycan isoform distribution) is high-performance liquid chromatography (HPLC) coupled to mass spectrometry (MS) [12–15]. LC-MS approaches to study protein glycosylation can be categorized as glycoprotein- or glycopeptide-based analysis. The former begins with enrichment of glycoproteins followed by protein digestion and LC-MS analysis [15–18], while in the latter, glycoproteins are initially digested and the resulting mixture is enriched at the glycopeptide level [15, 19–21]. Although recent advances in mass spectrometry have made large-scale identification of proteins feasible, it is still very challenging to analyze protein glycosylation in complex samples. This is due to the fact that glycopeptides often constitute a minor portion of the total peptide mixture, that signal intensity of glycopeptides is often low compared to nonglycosylated peptides and that the signal is often suppressed in the presence of other peptides [22–24]. For this reason, glycopeptide enrichment and separation is of main importance when performing glycoproteomic studies. Several excellent reviews partly or fully devoted to glycoproteomics based on separation or enrichment of glycopeptides have been published [20, 25–32].

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Abbreviations: **CID**, collision-induced dissociation; **Click OEG-CD**, click β-cyclodextrin oligo (ethylene glycol); **Con A**, concanavalin A; **ERLIC**, electrostatic repulsion hydrophilic interaction chromatography; **ETD**, electron transfer dissociation; **HILIC**, hydrophilic interaction chromatography; **IGOT**, isotope-coded glycosylation-site-specific tagging; **PGC**, porous graphitized carbon; **RNase B**, ribonuclease B; **SCX**, strong cation exchange; **TIMP-1**, tissue inhibitor of metalloproteinase-1; **TiO2**, titanium dioxide; **WGA**, wheat germ agglutinin; **ZIC-HILIC**, zwitterionic HILIC

However, in most cases they cover only a part of the known methods.

In this review, we will highlight the different approaches that have been developed for the enrichment of glycopeptides from complex samples to unravel the glycoproteome.

2 Methods based on lectin affinity enrichment

Lectins form a diverse group of proteins that recognize glycan structures attached to glycoproteins. Lectins were originally discovered due to their activity to agglutinate cells and the carbohydrate-binding activity of most of them resides in a limited polypeptide segment. The chemical groups involved in their attachment to glycans are very diverse including backbone amino groups, amides, aliphatic, and phenolic hydroxyls, hydrophobic residues, as well as metal ions. In general, binding takes place through hydrogen bonds with added contributions from van der Waals contacts and hydrophobic interactions [33]. In the last decades, this carbohydrate-binding capacity has been widely exploited for the isolation of glycoproteins [16, 34–39] or glycopeptides from complex samples [34, 40, 41]. Table 1 gives an overview over the different lectin affinity strategies that have been employed for glycopeptide enrichment.

For glycopeptide enrichment from complex samples, lectins have been used in different formats. These formats include agarose/sepharose-conjugated lectins packed in centrifugal devices, spin or low-pressure LC columns [17, 42–67] as well as lectins conjugated to HPLC-compatible matrices that enable high-pressure/high flow rate chromatography [68–72]. Common to all formats is that glycopeptides carrying the glycan structures that have affinity toward the selected lectin are retained on the lectin-supports and nonglycosylated peptides or nontargeted glycopeptides flow through. The bound fraction containing the enriched glycopeptides is subsequently eluted employing specific mono- or disaccharides that compete with the glycopeptide for binding to the lectin [17, 42, 44–49, 51, 53–55, 57–61, 63–66, 70–76], or by employing acidic conditions that disrupt the glycopeptide–lectin interaction [62, 67–69, 77, 78]. Recently, the use of lectins in solution has also been described [77, 79, 80]. In this approach, the sample is added on top of a centrifugal device where the ultrafiltration membrane allows separation of proteins from detergents, salts, and small molecular weight reagents from the proteins. Protein digestion is performed on the filter membrane followed by the addition of a free lectin solution. The glycosylated peptides are bound to the lectin and thereby retained by the filter (recommended cut-off <50 kDa [81]) whereas nonglycosylated peptides can be washed through by centrifugation. Next, glycopeptides are released by deglycosylation with PNGase F [79–81], consequently losing the glycan information. In addition, Bedair et al. [82] have developed a lectin-porous monolith sprayer that allows the preconcentration of glycopeptides in the electrospray emitter. In this setup, tryptic digests can be pumped offline into the lectin-porous monolith sprayer and washed. The emitter is then mounted onto the MS and the enriched glycopeptides are eluted by the infusion of an acidic solution.

Currently, there are many commercially available lectins that may be employed for the selective enrichment of glycopeptides [34, 40]. These lectins may have a broad specificity, such as the commonly employed Concanavalin A (Con A) that recognizes oligomannosyl motifs in *N*-linked glycans [83], as well as, narrower specificity such as *Aleuria alantia* lectin, or *Sambucus nigra* agglutinin that recognize fucose- [84] or sialic acid- [85] containing oligosaccharides, respectively. A limitation of lectin affinity chromatography for glycoproteomics derives from the fact that an individual lectin binds to a given glycan motive. Thus, when only a given lectin (single lectin chromatography) is used for enrichment, only a subset of glycopeptides will be captured reducing protein or peptide coverage. To overcome this limitation, a series of lectins (serial lectin affinity enrichment introduced by Cummings et al. [86]) [46, 51, 58] as well as mixtures of lectins (multilectin affinity enrichment introduced by Hancock and coworkers [87]) [49, 79, 80, 88] may be employed. In addition, extra enrichment or fractionation steps such as hydrophilic interaction chromatography (HILIC) [49, 50, 70], or lectin affinity enrichment at two levels (glycoprotein and glycopeptide level, or twice at the glycopeptide level) [17, 49, 50, 54, 61, 63] may be used to achieve greater protein or peptide coverage as well as to reduce nonspecific binding.

A commonly employed strategy in combination with glycopeptide lectin enrichment for glycosylation site identification is the use of PNGase F for deglycosylation in the presence of $H_2{}^{18}O$ [17, 44, 45, 48, 51, 53, 63, 70, 76]. This strategy was introduced by Gonzalez et al. [89] and adopted by Kaji and coworkers to develop the isotope-coded glycosylation site-specific tagging strategy [63, 90]. *N*-Glycan release by PNGase F turns asparagine into aspartic acid causing a one mass unit shift. This mass shift may be detected by mass spectrometry allowing the localization of the original glycosylation site. However, deamidation of asparagine to aspartic acid can also happen independent of enzymatic deglycosylation leading to the incorrect assignment of glycosylation sites [91]. Although it may not be used as a unique criterion, PNGase F peptide deglycosylation may be performed in $H_2{}^{18}O$ to improve the confidence in glycosylation site assignment.

To date, most of the work using lectin affinity for glycopeptide enrichment has focused on *N*-glycosylation. The problem of using lectins for studying *O*-glycosylation is that they are not sufficiently specific [46, 52, 71]. Jacalin is a common lectin employed to enrich *O*-glycopeptides since it binds to the GalNAc core found in *O*-glycosylation [46, 71]. However, the degree of nonspecific binding of this lectin as well as its cross-reactivity with high mannose *N*-glycopeptides is significant [46, 71]. Durham et al. have shown that this can be solved by the employment of serial lectin chromatography where a Con A affinity step before Jacalin selection greatly improved *O*-glycopeptide specificity [71].

Table 1. Lectin affinity enrichment of glycopeptides

Lectin	Support	Lectin enrichment	Sample	Sample treatment	Elution	LC-MS	Labeling	Objective	References
Con A	Lectin-bound agarose column	Single	Rat liver tissue	Protein extraction from tissue/trypsin digestion/glycopeptide lectin enrichment/desalting/glycopeptide deglycosylation (Endo H and PNGase F)	Saccharide solution	nanoLC-ESI MS	Label free	Comparison of two enzymatic deglycosylation strategies for *N*-glycosylation site assignment	[42]
SNA AAL	Lectin-bound POROS-AI column	Single	Human plasma	Multiple affinity removal system (MARS)-14 / trypsin digestion/desalting/glycopeptide lectin enrichment/glycopeptide deglycosylation (PNGase F)	Acid conditions	nano2D- LC ESI-MS MALDI-MS	Label free	Enrichment of glycosites containing sialic acid or fucose (increases in sialylation and fucosylation are cancer-associated modifications) from digested human plasma	[68]
Con A WGA	Lectin isolation kit	Single and multiple	Human plasma (patients with hepatocellular carcinoma and healthy individuals)	MARS-7/trypsin digestion/glycopeptide lectin enrichment/glycopeptide deglycosylation (PNGase F)/2D LC (strong cation exchange + reverse phase) peptide fractionation	According to manufacturer's protocol	nanoLC-ESI-MS MALDI-MS	Label free	Development of a glycoproteomics workflow for biomarker discovery in hepatocellular carcinoma The capability of hydrazide chemistry and lectin affinity for the enrichment of glycopeptides is compared	[88]
Con A WGA RCA_{120} SSA MAM	Free lectin solution	Single	Tryptic digest of glycoproteins (Ribonuclease B, ovalbumin, human serum transferrin)	Sample mixing with lectin solution/transfer to centrifugal tube and centrifugation/retentate elution with 10 mM HCl and centrifugation	Acid conditions	UPLC-ESI-MS	Label free	Development of an affinity entrapment method for oligosaccharides and glycopeptides using a free lectin solution	[77]
ConA WGA RCA_{120}	Free lectin solution	Single and multiple	Mouse tissues (brain, liver, kidney, heart) and blood plasma	Tissue dissection/protein extraction/FASP method/peptide Stage Tips purification	PNGase F digestion ($H_2{}^{18}O$)	nanoLC-ESI-MS	Label free	Mapping of *N*-glycosylation sites in mouse tissue and blood plasma using *N*-glyco FASP	[80]
ConA WGA RCA_{120}	Free lectin solution	Multiple	Mouse liver samples	Tissue dissection, fixation and microdissection/protein extraction/*N*-glycoFASP/Stage Tip peptide fractionation	PNGase F digestion ($H_2{}^{18}O$)	nanoLC-ESI-MS	Label free	Development of a protocol for glycoproteomic analysis of formalin-fixed and paraffin-embedded tissue samples using *N*-glyco FASP	[79]
Con A	Lectin-bound agarose column	Single	Serum samples (COPD and lung adenocarcinoma patients, and healthy controls)	MARS-14/Trypsin digestion/desalting/8-plex iTRAQ labeling/glycopeptide lectin enrichment/strong cation exchange chromatography	PNGase F digestion ($H_2{}^{18}O$)	nanoLC-ESI-MS	8-plex iTRAQ	Simultaneous identification of glycosylation sites and site-specific quantification of glycan structure changes among COPD, lung adenocarcinoma patients, and healthy individuals	[43]
SNA	Lectin-bound agarose centrifugal columns	Single	Tryptic digest of bovine fetuin and serum samples (prostate cancer patients and healthy individuals)	IgG removal using protein AG beads (for serum samples)/trypsin digestion/glycopeptide lectin-enrichment/acetylation with ($^1H_3/^2D_3$)/glycopeptide deglycosylation (PNGase F, $H_2{}^{18}O$)/C-18 chromatography purification	Saccharide solution	nanoLC-ESI-MS	acetylation with ($^1H_3/^2D_3$)	Method development for comprehensive comparative analysis of sialylated glycopeptides and glycosylation sites in a model protein and healthy and prostate cancer serum samples	[44]

Table 1. Continued

Lectin	Support	Lectin enrichment	Sample	Sample treatment	Elution	LC-MS	Labeling	Objective	References
Con A	Lectin-bound sepharose disposable column	Single	Cell culture (pluripotent murine embryonic stem cells, ES, and ES cells differentiated into embroid bodies)	Cell harvest/protein extraction/trypsin digestion/desalting/glycopeptide lectin enrichment/desalting/glycopeptide deglycosylation (PNGase F, $H_2{}^{18}O$)/filtration	Saccharide solution	LC-ESI-MS	Label free	Protocol development for the identification of potential glycoprotein biomarkers that express specific *N*-linked glycans in specific glycosylation sites at defined developmental stages during embryonic stem cell differentiation.	[45]
Jacalin	Lectin-bound agarose column	Single and serial	Bovine serum	Single enrichment: trypsin digestion/desalting/glycopeptide lectin enrichment/desalting Double enrichment: glycoprotein lectin enrichment/trypsin digestion/desalting/glycopeptide lectin enrichment/desalting	Saccharide solution	nanoLC-ESI- MS	Label free	Analysis of secreted *O*-linked glycopeptides	[46]
AAL	Lectin-bound agarose column	Single	Murine kidney cells	Protein extraction/trypsin digestion/glycopeptide lectin enrichment/desalting	Saccharide solution	LC-ESI-MS	Label free	Method development for the identification and screening of glycoproteins carrying Lewis epitopes	[47]
SNA AAL	Lectin-bound POROS column	Single	Human plasma	MARS-14/Trypsin digestion/glycopeptide lectin enrichment/desalting/glycopeptide deglycosylation (PNGase F)	Acid conditions	LC-ESI-MS	Label free	Written and video protocol for isolation and identification of glycopeptides carrying specific glycans from human serum.	[69]
Con A	Lectin-bound sepharose disposable column	Single	Stem cell pellets (murine stem cells and embroid bodies pellets)	Protein extraction/trypsin digestion/desalting/glycopeptide lectin enrichment/desalting/glycopeptide deglycosylation (PNGase F, $H_2{}^{18}O$)	Saccharide solution	LC-ESI-MS	Label free	Glycoproteomic analysis of embryonic stem cells	[48]
Con A SNA WGA	Lectin-bound agarose microcolumn	Single and multiple	Venous blood samples (healthy controls and women with breast cancer)	Glycoprotein lectin enrichment/trypsin digestion/glycopeptide enrichment (lectin affinity or HILIC)/glycopeptide deglycosylation (PNGase F)	Saccharide solution	MALDI- MS and nanoLC-ESI- MS	Label free	Development of a protocol to recover and identify serum glycoproteins and characterize their *N*-glycosylation sites. Different enrichment strategies based on lectin affinity (at glycopeptide and glycoprotein level) and/or HILIC were tested	[49]
SNA Con A	Lectin-bound agarose column	Serial	Lys-C digests of human serum transferrin and bovine pancreatic ribonuclease B	Lys-C digestion/cellulose affinity enrichment/glycopeptide SNA affinity enrichment/glycopeptide ConA affinity enrichment/cellulose affinity enrichment	Guanidine thiocyanate solution	MALDI- MS	Label free	Development of a rapid and sensitive method for analyzing glycopeptides by introducing lectin and cellulose column chromatography in combination with MALDI-TOF-MS	[50]

Table 1. Continued

Lectin	Support	Lectin enrichment	Sample	Sample treatment	Elution	LC-MS	Labeling	Objective	References
Con A	Con A packed TopTips	Single	Model glycoproteins (immunoglobulin G, transferrin, α1-acid glycoprotein)	Trypsin digestion/glycopeptide enrichment (HILIC, lectin affinity, or reversed-phase HPLC)/glycopeptide deglycosylation (PNGase F)	Saccharide solution	MALDI- MS	Label free	In-depth study of maximizing glycosylation coverage on model glycoproteins by optimizing all the aspects of glycopeptide-based analysis, including sample preparation methods, mass spectral techniques, and data analysis strategies. For sample preparation, several approaches, including reversed-phase HPLC, lectin affinity, and HILIC were compared and tested individually as well as in parallel.	[73]
GaL6 Con A WGA	Lectin-bound TSK GEL column	Single	*C. elegans* cell culture	*C. elegans* lysis/protein extraction/trypsin digestion/glycopeptide lectin enrichment/glycopeptide HILIC enrichment/glycopeptide deglycosylation (PNGase F, $H_2{}^{18}O$)	Saccharide solution	nanoLC-ESI-MS	Label free	Analysis and characterization of glycoprotein expression in *C. elegans*	[70]
Con A	Lectin-bound agarose column	Single	Human serum samples (healthy and pancreatic cancer serum)	Glycoprotein enrichment/trypsin digestion/glycopeptide enrichment/glycopeptide deglycosylation (PNGase F, $H_2{}^{18}O$)/RP-HPLC for glycopeptide analysis and HILIC for glycan analysis	Saccharide solution	LC-ESI-MS	Label free	Method development for the comprehensive analysis of *N*-glycans and glycosylation sites in human serum samples.	[17]
Con A WGA SNA WFA	Lectin-bound agarose columns	Single and serial	*Drosophila melanogaster* heads	Head collection and homogenization/ protein extraction/trypsin digestion/glycopeptide lectin-enrichment/ glycopeptide deglycosylation (PNGase F, $H_2{}^{18}O$)	Saccharide solution	LC-ESI-MS	Label free	Identification of *N*-glycosylated proteins from the central nervous system of *Drosophila melanogaster*	[51]
WGA	Lectin-bound agarose column	Single	Postsynaptic density preparation	Trypsin digestion/desalting/ glycopeptide lectin enrichment/ β-elimination-michael addition/ desalting	-	nanoLC-ESI-MS	Label free	Development of a method for direct enrichment and identification of *in vivo O*-GlcNAc-modified peptides through lectin affinity and mass spectrometry	[52]
Con A	Lectin-bound sepharose spin column	Single	*Trypanosoma cruzi* trypomastigotes	Trypomastigotes harvest/cell lysis/ subcellular fractionation/protein extraction/trypsin digestion/ desalting/glycopeptide lectin enrichment/desalting/ glycopeptide deglycosylation (PNGase F, $H_2{}^{18}O$)	Saccharide solution	LC-ESI-MS	Label free	Glycoproteomic analysis of *Trypanosoma cruzi* trypomastigotes	[53]
Con A Jacalin	Lectin-bound silica column	Serial	Bovine fetuin and human sera	Trypsin digestion/neuraminidase digestion/Con A high-mannose glycopeptide removal/Jacalin *O*-glycopeptide enrichment/chemical deglycosylation	Saccharide solution	LC-ESI-MS and MALDI-MS	Labeling with (d_3)N-acetoxysuccinamide	Development of a serial lectin affinity approach for the study of *O*-glycosylated proteins from the human blood proteome	[71]

Table 1. Continued

Lectin	Support	Lectin enrichment	Sample	Sample treatment	Elution	LC-MS	Labeling	Objective	References
Con A	Lectin-bound agarose column	Single	Human platelets	Platelets lysis/glycoprotein lectin enrichment/trypsin digestion/glycopeptide lectin enrichment/glycopeptide deglycosylation (PNGase F)	Saccharide solution	nanoLC-ESI-MS	Label free	Elucidation of *N*-glycosylation sites on human platelet proteins	[54]
Con A	Lectin-bound sepharose column	Single	Recombinant human thyrotropin	Trypsin digestion/glycopeptide lectin enrichment/desalting/RP-HPLC	Saccharide solution	MALDI-MS	Label free	Characterization of recombinant human thyrotropin glycosylation	[55]
Con A	Lectin-bound monolith sprayer	Single	Ribonuclease B	Trypsin digestion/glycopeptide lectin enrichment	Acid conditions	ESI-MS	Label free	Application of a porous polymer monolith nanospray emitter for the enrichment and MS analysis of glycopeptides from ribonuclease B tryptic digest	[82]
Con A	Lectin-bound agarose column	Single	Epidermis and dermis samples	Tissue deffating/lyophilization/trypsin digestion/glycopeptide lectin enrichment/HPLC separation/glycopeptide deglycosylation (PNGase F)	Saccharide solution	MALDI-MS	Label free	Approach development for the glycoproteome study of the murine epidermis	[56]
Con A SNA	Lectin-bound silica micro-columns	Single	Fetuin	Trypsin digestion/on-line glycopeptide enrichment/desalting/LC-MS analysis	Saccharide solution	LC-ESI-MS	Label free	Development of lectin microcolumns for on-line lectin enrichment, desalting, and LC-MS analysis	[72]
SNA Con A	Lectin-bound agarose column	Single and serial	Human serum	Trypsin digestion/peptide acetylation/glycopeptide lectin enrichment/HPLC fractionation/glycopeptide deglycosylation (PNGase F)	Saccharide solution	ESI-MS	Labeling with *N*-acetoxy-(d_3)-succinimide	Comparison of the relative degree of sialylation among human serum glycoproteins	[58]
SNA Con A	Lectin-bound agarose column	Serial	Human serum	Trypsin digestion/peptide acetylation/glycopeptide lectin enrichment/HPLC fractionation/glycopeptide deglycosylation (PNGase F)	Saccharide solution	ESI-MS	Labeling with *N*-acetoxy-(d_3)-succinimide	Comparison of sialylated protein glycoforms containing differentially branched complex-type glycans	[57]
Con A	Lectin-bound column	Single	*C. elegans* worms	Protein extraction/trypsin digestion/glycopeptide lectin enrichment/glycopeptide deglycosylation (PNGase F)	Saccharide solution	MALDI -MS Off-line LC-MALDI-MS 2D-LC–ESI-MS	Label free	Identification of the hydrophobic glycoproteins of *C. elegans*	[59]
Con A	Lectin-bound sepharose column	Single	Human plasma-derived antithrombin	Trypsin digestion/glycopeptide lectin enrichment	Saccharide solution	ESI- MS MALDI- MS	Label free	Characterization of human plasma-derived antithrombin glycosylation	[60]
Con A WGA	Lectin-bound agarose column	Single	Human serum	Glycoprotein lectin enrichment/Lys-C digestion/desalting/glycopeptide lectin enrichment/ glycopeptide deglycosylation (PNGase F)/trypsin digestion	Saccharide solution	LC-ESI-MS	Label free	Development of a method for extensive profiling of *N*-glycosylated proteins	[61]
Con A	Lectin-bound column	Single	*C. elegans* worms	Membrane protein solubilization and precipitation/trypsin digestion/glycopeptide lectin enrichment/glycopeptide deglycosylation (PNGase F)	Acid conditions	MALDI-MS	Label free	Method development for the identification of membrane-bound proteins containing *N*-glycans *C. elegans* worms	[62]

Table 1. Continued

Lectin	Support	Lectin enrichment	Sample	Sample treatment	Elution	LC-MS	Labeling	Objective	References
LTA	Lectin-bound silica column	Single	Serum samples from dogs with lymphosarcoma at various stages of treatment	Trypsin digestion/peptide acetylation/glycopeptide lectin enrichment/HPLC glycopeptide fractionation/glycopeptide deglycosylation (PNGase F)	Saccharide solution	MALDI-MS ESI-MS	Labeling with *N*-acetoxy-(d_3)-succinimide	Development of a method based on lectin selection and isotope coding for comparative proteomics of glycoproteins	[74]
Con A	Lectin-bound agarose column	Single	*C. elegans* worms	Worm lysis/protein extraction/glycoprotein lectin enrichment/trypsin digestion/glycopeptide lectin enrichment/glycopeptide deglycosylation (PNGase F, $H_2{}^{18}O$)/ 2D LC (anion exchange + reverse phase) peptide fractionation	Saccharide solution	LC-ESI-MS	Label free	Description of IGOT strategy and its application to the characterization of *N*-linked glycoproteins from *C. elegans*	[63]
Con A GaL6	Lectin-bound agarose column	Serial	*C. elegans* worms	Worm lysis/protein extraction/glycoprotein lectin enrichment/Lys C digestion/glycopeptide lectin enrichment/RP-HPLC fractionation	Saccharide solution	Protein sequencer	Label free	Approach development for the analysis of soluble glycoproteins produced in *C. elegans*	[64]
LTA	Lectin-bound silica column	Single	Model proteins (human lactoferrin, bovine fetuin) and human sera	Trypsin digestion/glycopeptide lectin enrichment/RP-HPLC fractionation/glycopeptide deglycosylation (PNGase F, $H_2{}^{18}O$)	Saccharide solution	MALDI-MS ESI-MS	Label free	Method development for the identification of glycoproteins with particular types of glycosylation using lectin affinity enrichment	[76]
Con A BS-II	Lectin-bound silica column	Single	Human serotransferrin and human serum	Trypsin digestion/glycopeptide lectin affinity enrichment/RP-HPLC fractionation/(glycopeptide deglycosylation (PNGase F))	Saccharide solution	MALDI-MS	Label free	Glycoprotein analysis based on lectin-selection of glycopeptides from tryptic digests	[75]
Con A RCA I SBA E4-PHA WGA LCA UEA	Lectin-bound agarose or sepharose columns	Serial	Rat liver samples	Protein extraction/immunoaffinity chromatography/preparative electrophoresis/pronase digestion/acetylation with (^{14}C) acetic anhydride/serial lectin glycopeptide enrichment	Saccharide solution	—-	Acetylation with (^{14}C) acetic anhydride	Characterization of rat liver microsomal and lysosomal β-glucuronidase-derived glycopeptides	[65]
Con A BS-II	Lectin-bound column	Single	Human serotransferrin and human serum	Trypsin digestion/glycopeptide lectin enrichment/RP-HPLC fractionation/peptide acetylation	Saccharide solution	MALDI-MS ESI-MS	Labeling with *N*-acetoxy-(d_3)-succinimide	Strategy development for qualitative and quantitative analysis of signature peptides	[66]
Con A WGA PNA	Lectin-bound agarose column	Serial	Cell line	Cell lysis/immunoaffinity isolation of peptides from human class I MHC molecules/glycopeptide lectin affinity/(Galactosyltranferase-mediated labeling of peptides bounding to WGA)/RP-HPLC fractionation	Acid conditions	Protein sequencer	Label-free and galactosyltranferase-mediated labeling of peptides bounding to WGA	Study of the natural presentation of glycosylated peptides by class I MHC molecules	[67]

AAL, *Aleuria alantia* lectin; Con A, concanavalin A; HILIC, hydrophilic interaction chromatography; IGOT, isotope-coded glycosylation site-specific tagging; LCA, *Lens culinaris* agglutinin; LTA, *Lotus tetragonolobus* agglutinin; MAM, *Maackia amurensis* agglutinin; MARS, multiple affinity removal system; PHA, phitohemaglutinin; PNA, peanut agglutinin; RCA, *Ricinus communis* agglutinin; SBA, soybean agglutinin; SNA, *Sambucus nigra* agglutinin; SSA, *Sambucus sieboldiana* lectin; UEA, *Ulex europaeus* agglutinin; WFA, *Wisteria floribunda*; WGA, wheat germ agglutinin.

3 Methods based on covalent interactions: hydrazide and boronic acid chemistry

In this section, the strategies for glycopeptide enrichment based on covalent bond formation between the targeted glycopeptides and functionalized solid supports are reviewed and summarized. These methods can be classified into two categories: those based on hydrazide chemistry and those based on boronic acid interactions.

3.1 Hydrazide chemistry

In 2003, Zhang et al. [92] developed a solid-phase extraction method for isolating glycoproteins based on hydrazide chemistry, which has been widely employed for glycopeptide enrichment (see Table 2). The methodology involves the following steps (see Fig. 1):

(i) Oxidation of carbohydrate cis-diol groups to aldehydes with periodate.
(ii) Hydrazone formation between aldehydes and hydrazide groups immobilized on a solid support. This allows the covalent immobilization of glycoproteins while nonglycosylated proteins can be removed by washing.
(iii) Digestion of the immobilized glycoproteins generally employing trypsin. This allows the removal of nonglycosylated peptides whereas glycopeptides are still retained on the solid support.
(iv) Release of glycopeptides from the solid support by digestion with PNGase F.

The same research group optimized the original method and modified it for glycopeptide isolation from tryptic digests. In the modified method, glycoproteins are first digested into a mixture of peptides and glycopeptides followed by oxidation of glycopeptides with periodate and capture on the hydrazide support [93]. The modified procedure resulted in higher specificity for glycopeptide capture when compared to hydrazide-based glycopeptide enrichment performed at the glycoprotein level [93–95]. This may be due to the fact that digestion of proteins into peptides prior to capture increases the accessibility of *N*-glycopeptides to the hydrazide resin increasing the capturing efficiency [93, 96]. However, enrichment at the glycoprotein level may result in a higher number of glycopeptide/glycoprotein identifications [94, 95]. Thus, performing the enrichment at the peptide or protein level, or both, will depend on the specific research question. Additionally, for comparative quantitative MS analysis, the enriched glycopeptides can be labeled with isotopic tags (e.g. d0/d4 succinic anhydride, iTRAQ) [92, 93, 97–102], or deglycosylated in the presence of $H_2{}^{16}O/H_2{}^{18}O$ [103].

Although conceptually both *N*- and *O*-glycoproteins/glycopeptides can be captured using this method, further analysis of the enriched glycopeptides is practically limited to *N*-glycopeptides since there is a lack of efficient enzymes that can specifically release *O*-linked glycopeptides from the solid support [104]. Chemical approaches for the removal of *O*-linked carbohydrates, such as the use of hydroxylamine may be employed [104].

Generally, hydrazide-terminated resins or gels are employed for glycopeptide enrichment [54, 92, 105–111]. However, in order to make this extraction method suitable for high-throughput analysis, magnetic particles may be synthesized [112] or modified with hydrazide chemistry [94, 95, 103, 113].

Due to the high specificity of the hydrazide capture method [101], usually glycoprotein identification relies on a limited number of peptides (on average one or two glycopeptides from each glycoprotein are captured [114]) after deglycosylation. Thus identified proteins have low-sequence coverage, and most of them are identified based on a single peptide. Glycoprotein coverage can be improved by the analysis of the nonglycopeptides produced by trypsin digestion [98, 115] (when the method is employed for glycoprotein capture) or by using a two-step protease digestion [116] (glycopeptides are generated employing Lys-C, captured, and further digested with trypsin, then the nonglycopeptides produced by Lys-C + trypsin are analyzed as well as the PNGase released glycopeptides). Glycoproteome/glycosite coverage may also be improved through a multiple-enzyme digestion strategy [117], or by the combination of different enrichment techniques [97, 108, 115, 118–120]. In addition, the sensitivity of the method may be increased by combining hydrazide chemistry with high-abundant protein depletion or by employing two-dimensional chromatographic separations [95, 105].

While this method allows identification of the glycosylation sites, the oxidative chemical coupling of the glycans to the hydrazide support and the subsequent glycopeptide release with PNGase F does not provide information about the glycan structures or the degree of site occupancy. To overcome this hurdle, Nilsson and coworkers modified the hydrazide methodology for the selective enrichment of sialic acid-containing glycopeptides [121]. The strategy is based on the fact that sialic acid-containing glycopeptides can be selectively oxidized by mild periodate oxidation, bound onto hydrazide supports, and released by acid hydrolysis without the need of PNGase F. This allows maintenance of the glycopeptide glycan moiety with the exception of the sialic acid. However, this method does not allow the enrichment of the nonsialic acid-containing glycopeptides and sialylation cannot be studied.

Hydrazide capture has been applied for the analysis of glycoproteins in complex mixtures such as serum/plasma and other body fluids [88, 92, 94, 95, 99, 102, 103, 105, 106, 112, 119, 122–128], as well as for the analysis of secreted [114, 118, 120], membrane-bound [107, 115, 129], tissue [97, 109, 117, 130], or cell lysate-derived [98, 116, 131] glycoproteins. In addition, hydrazide capture has been adapted for the selective identification of cell surface glycoproteins (cell surface capturing technology [100, 132–134]). The steps of this strategy involve (i) oxidation of carbohydrate-containing proteins on living cells, (ii) gentle, covalent chemical labeling of the

Table 2. Hydrazide chemistry for glycopeptide enrichment

Glycoprotein/ glycopeptide level	Support	Sample	Sample treatment	LC-MS	Labeling	Objective	References
Glycopeptide	Hydrazide resin	Rat myocardial tissue	Protein extraction from tissue / protein digestion (trypsin, endoproteinase Asp-N, thermolysin) / peptide dephosphorilation / iTRAQ labeling / peptide oxidation / mixture with hydrazide resin / washing / glycopeptide release with PNGase F	LC-ESI-MS	iTRAQ labeling	Quantitative *N*-glycoproteomics of rat myocardial tissue employing digestion with multiple proteases and parallel glycopeptide enrichment strategies (hydrazide capture, titanium oxide, and HILIC)	[97]
Glycopeptide and glycoprotein	Hydrazide-modified magnetic beads	Mouse plasma, *Aspergillus niger* secretome and whole cell lysate	Glycopeptide level: protein extraction / protein digestion (trypsin) / peptide oxidation / mixture with hydrazide magnetic beads / washing / glycopeptide release with PNGase F Glycoprotein level: protein extraction / protein oxidation / mixture with hydrazide magnetic beads / washing / protein digestion (trypsin) / washing / glycopeptide release with PNGase F	LC-ESI-MS	Label free	Mapping *N*-linked glycosylation sites in the secretome and whole cells of *Aspergillus niger*. Hydrazide chemistry at the glycoprotein and peptide-level strategies are compared	[94]
Glycopeptide	Hydrazide resin	Chardonay white wine	Protein extraction / protein digestion (trypsin) / peptide oxidation / mixture with hydrazide resin / washing / glycopeptide release with PNGase	MALDI-MS nanoLC-ESI-MS	Label free	Investigation of the wine glycoproteome employing different glycopeptide enrichment strategies (HILIC, TiO_2, and hydrazide capture)	[108]
Glycopeptide	Hydrazide gel	Rat brain tissue	Protein extraction / protein digestion (trypsin) / peptide oxidation / mixture with hydrazide gel / washing / glycopeptide release with PNGase F / 2D LC (strong cation exchange + reverse phase) peptide fractionation	nanoLC-ESI-MS	Label free	Characterization of insoluble and soluble glycoprotein fractions in rat brain tissue	[109]
Glycopeptide	Hydrazide gel	Cell lysate	Protein extraction / protein digestion (Lys-C) / peptide oxidation (10 mM $NaIO_4$) / mixture with hydrazide resin / washing / peptide digestion (trypsin) for the release of LT-peptides / glycopeptide release with PNGase F	MALDI-MS	Label free	Development of a two-step protease digestion and glycopeptide capture approach for glycosite identification and glycoprotein sequence coverage improvement	[116]
Glycopeptide	Hydrazide resin	Zebra fish embryos	Protein extraction / protein digestion (trypsin / chymotrypsin) / peptide oxidation / mixture with hydrazide resin / washing / glycopeptide release with PNGase F	nanoLC-ESI-MS	Label free	Identification of specific *N*-glycosylated proteins and *N*-linked glycosites of zebra fish	[111]

Table 2. Continued

Glycoprotein/ glycopeptide level	Support	Sample	Sample treatment	LC-MS	Labeling	Objective	References
Glycoprotein	Hydrazide gel	Human plasma samples (healthy individuals, hepatocellular carcinoma patients)	MARS-7 depletion / protein oxidation / mixture with hydrazide resin / protein digestion (trypsin) / washing / glycopeptide release with PNGase F / 2D LC (strong cation exchange + reverse phase) peptide fractionation	nano-LC-ESI-MS MALDI-MS	Label free	Development of a glycoproteomics workflow for biomarker discovery in hepatocellular carcinoma The capability of hydrazide chemistry and lectin affinity for the enrichment of glycopeptides is compared	[88]
Glycoprotein	Hydrazide resin	Cow saliva	Protein extraction / protein oxidation / mixture with hydrazide resin / washing / protein digestion (trypsin) / washing / glycopeptide release with PNGase F	LC-ESI-MS	Label free	Development of a three-step approach (nontargeted, targeted, and glycocapture) for the study of the bovine salivary proteome	[125]
Glycoprotein	Hydrazide resin	Serum samples (lung cancer patients and controls)	MARS-14 depletion / protein oxidation / mixture with hydrazide resin / washing / protein digestion (trypsin) / washing / glycopeptide release with PNGase F	nanoLC-ESI-MS	Label free	Employment of hydrazide capture for lung cancer biomarker discovery	[124]
Glycoprotein	Hydrazide resin	*Drosophila melanogaster* proteasome complex	Protein oxidation / mixture with hydrazide resin / washing / protein digestion (trypsin) / washing / *O*-glycopeptide release with hydroxylamine hydrochloride / β-elimination-michael addition	MALDI-MS LC-ESI-MS	Label free	Development of a method for the enrichment of *O*-Glc NAc-modified proteins employing the hydrazide capture strategy	[104]
Glycopeptide	Hydrazide gel	Mouse brain	Membrane protein extraction / protein digestion (trypsin) / peptide oxidation / mixture with hydrazide gel / washing / glycopeptide release with PNGase F	LC-ESI-MS	Label free	Characterization of glyco- and phosphoproteomes from mouse brain membranes. Electrostatic repulsion hydrophilic interaction chromatography and hydrazide capture are compared.	[129]
Glycopeptide	Hydrazide gel	Metastasic cell lines (PC3 and LNCaP)	Cell lysis / protein extraction / internal standard spiking / protein digestion (trypsin) / isotope coded protein labeling / peptide oxidation / mixture with hydrazide resin / washing / glycopeptide release with PNGase F / 2D LC (strong cation exchange + reverse phase) peptide fractionation	LC-ESI-MS	ICPL labeling	Method development for quantitative profiling of phospho- and glycoproteins originating from metastatic carcinoma cell lines	[98]
Glycopeptide and glycoprotein	Hydrazide-modified magnetic beads, hydrazide resin	Human plasma	Glycopeptide level: (MARS-14 depletion) / protein digestion (trypsin) / peptide oxidation / mixture with hydrazide support / washing / glycopeptide release with PNGase F / RP-HPLC fractionation Glycoprotein level: (MARS-14 depletion) / protein oxidation / mixture with the hydrazide support / washing / protein digestion (trypsin) / washing / glycopeptide release with PNGase F / RP-HPLC fractionation	nanoLC-ESI-MS	Label free	Development and optimization of a protocol for glycopeptide capture from plasma employing hydrazide-modified supports A comparison of the glycocapture method at the glycoprotein and glycopeptide level is performed	[95]

Table 2. Continued

Glycoprotein/ glycopeptide level	Support	Sample	Sample treatment	LC-MS	Labeling	Objective	References
Glycopeptide	Hydrazide-modified magnetic beads	Lyophilized mouse serum	Serum spiking with standard glycoproteins / protein digestion (trypsin) / peptide oxidation / mixture with hydrazide-modified magnetic beads in the presence of ^{16}O- or ^{18}O-water and trypsin / washing / glycopeptide release with PNGase F in presence of ^{16}O- or ^{18}O-water	LC-ESI-MS	^{18}O labeling	Development of a strategy for the quantification of *N*-linked glycoproteins by $^{18}O_3$-labeling and hydrazide glycopeptide enrichment	[103]
Glycoprotein	Hydrazide-modified magnetic beads	Standard proteins (ribonuclease B, bovine fetuin, bovine serum albumin)	Glycoprotein enrichment: protein oxidation / mixture with hydrazide-modified magnetic beads / washing / PNGase F release of glycoproteins Glycopeptide enrichment: protein oxidation / mixture with hydrazide-modified magnetic beads / washing / protein digestion (trypsin) / washing / PNGase F release of glycopeptides	SDS-PAGE MALDI-MS LC-ESI-MS	Label free	Method development and comparison for the production of hydrazide-modified magnetic particles for glycopeptide / glycoprotein enrichment	[113]
Glycopeptide	Hydrazide gel	Mouse serum	Protein digestion (trypsin) / peptide oxidation / mixture with hydrazide resin / washing / asialoglycopeptide release with 1 M HCl / derivatization with 2-aminopyridine	LC-ESI-MS	Labeling with 2-aminopyridine	Development of a strategy for quantitative mouse serum glycoproteomics based on glycoblotting and focusing on sialic acid containing peptides	[123]
Glycoprotein on cell surface	Biocytin hydrazide	HL60 and NB4 cells	CSC variant 1: Cell oxidation / cell labeling with biocytin hydrazide / cell lysis and membrane preparation / protein digestion (trypsin) / streptavidin glycopeptide capture / washing / peptide elution: cysteine containing peptides (chemical reduction), and *N*-glycopeptides (PNGase F) CSC variant 2: Cell oxidation / cell labeling with sulfo-NHS-SS biotin or sulfo-NHS-LC-biotin / cell lysis and membrane preparation / protein digestion (trypsin) / streptavidin glycopeptide capture / washing / lysine containing peptide release (chemical reduction)	LC-ESI-MS	Label free	Application of complementary CSC variants for the identification and relative quantification of acute myeloid leukemia cell surface proteins	[135]
Glycopeptide	Hydrazide resin	Mouse tissue	Solubilization of solid tissues / protein digestion (trypsin) / peptide oxidation / mixture with hydrazide resin / washing / glycopeptide release with PNGase F	LC-ESI-MS	Label free	Extracellular glycoprotein profiling from mouse tissue	[130]

Table 2. Continued

Glycoprotein/ glycopeptide level	Support	Sample	Sample treatment	LC-MS	Labeling	Objective	References
Glycoprotein	Hydrazide resin	Thyroid cancer cell lines	Cell oxidation / cell lysis / spiking with periodate oxidized chicken albumin / mixture with hydrazide resin / washing / protein digestion (trypsin) / washing / glycopeptide release with PNGase F	LC-ESI-MS	Label free	Identification of cell surface and secreted candidate biomarkers for thyroid cancer	[131]
Glycoprotein	Hydrazide resin	Tear fluid (patients with climatic droplet keratopathy patients and healthy individuals)	Tear sample desalting / protein oxidation / mixture with hydrazide resin / washing / protein digestion (trypsin) / washing / glycopeptide release with PNGase F / iTRAQ labeling / 2D LC (strong cation exchange + reverse phase) peptide fractionation	nanoLC-ESI-MS	iTRAQ labeling	Quantitative analysis of *N*-linked glycoproteins in tear fluid	[99]
Glycoprotein	Hydrazide resin	U87MG cells treated with cAMP	Conditioned media collection / protein oxidation / mixture with hydrazide resin / protein digestion (trypsin) / glycopeptide release with PNGase F	nanoLC-ESI-MS	Label free	Identification of secreted *N*-linked glycoproteins regulated by cAMP in glioblastoma cells	[114]
Glycopeptide	Hydrazide gel	Influenza virus	Protein digestion (trypsin) / protein oxidation / mixture with hydrazide gel / washing / glycopeptide release with PNGase F	LC-ESI-MS	Label free	Glycosylation analysis of influenza virus samples	[110]
Glycoprotein	Hydrazide gel	HeLa cells	Cell oxidation / cell lysis / mixture with hydrazide gel / washing / protein digestion (trypsin) / washing / glycopeptide release with PNGase F	LC-ESI-MS	Label free	Identification of cell surface and secreted glycoproteins in HeLa cells	[118]
Glycoprotein	Hydrazide gel	Rat liver tissue	Membrane protein preparation / protein partitioning / protein oxidation / mixture with hydrazide gel / washing / protein digestion (trypsin) / washing / glycopeptide release with PNGase F	nanoLC-ESI-MS	Label free	Method development for the identification of rat liver membrane proteins based on their hydrophobicity and glycosylation	[115]
Glycopeptide	Hydrazide gel	Human liver tissue	Tissue homogenization / protein extraction / protein digestion (trypsin, pepsin, thermolysin) / peptide oxidation / mixture with hydrazide gel / washing / glycopeptide release with PNGase F / 2D LC (strong cation exchange + reverse phase) peptide fractionation	nanoLC-ESI-MS	Label free	Development of a strategy for the analysis of the *N*-glycoproteome of human liver tissue by the use of hydrazide chemistry in combination with a multiple enzyme digestion strategy	[117]
Glycopeptide	Hydrazide resin	Human breast cancer cell lines	Membrane preparation and protein extraction / protein digestion (trypsin) / peptide oxidation / mixture with hydrazide resin / washing / glycopeptide release with PNGase F	LC-ESI-MS	Label free	Site-mapping of *N*-glycosylated membrane proteins for breast cancer biomarkers	[107]

Table 2. Continued

Glycoprotein/ glycopeptide level	Support	Sample	Sample treatment	LC-MS	Labeling	Objective	References
Glycoprotein	Hydrazide resin	Human transferrin and human cerebrospinal fluid	Sialic acid oxidation / mixture with hydrazide resin / washing / protein digestion (trypsin) / washing / sialoglycopeptide release with formic acid	LC-ESI-MS	Label free	Enrichment of sialylated glycopeptides for glycan structure and attachment site identification	[121]
Glycoprotein on cell surface	Biocytin hydrazide	T, B, and embryonic stem cells	Cell oxidation / neuraminidase treatment (only in control experiments) / cell labeling with biocytin hydrazide / cell lysis and membrane preparation / protein digestion (LysC + trypsin) / streptavidin glycopeptide capture / washing / glycopeptide release with PNGase F	LC-ESI-MS	Label free	Employment of CSC technology for the detection and relative quantitative comparison of the cell surface *N*-glycoproteomes of T, B, and embryonic stem cells	[133]
Glycoprotein on cell surface	Biocytin hydrazide	Mouse myoblasts	Cell oxidation / cell labeling with biocytin hydrazide / cell lysis and membrane preparation / protein digestion (LysC + trypsin) / streptavidin glycopeptide capture / washing / glycopeptide release with PNGase F	LC-ESI-MS	Label free	Study of the cell surface *N*-linked glycoprotein subproteomes of myoblasts and identification of potential biomarkers of myoblast differentiation	[134]
Glycoprotein on cell surface and glycopeptide	Biocytin hydrazide and hydrazide resin	*Drosophila melanogaster* embryonic Kc167 cells	CSC: Cell oxidation / cell labeling with biocytin hydrazide / cell lysis and membrane preparation / protein digestion (trypsin) / washing / (labeling with isotope-coded protein label reagent) / streptavidin glycopeptide capture / washing / glycopeptide release with PNGase F Whole membrane glycocapturing: cell lysis and membrane preparation / trypsin digestion / peptide oxidation / mixture with hydrazide support / washing / glycopeptide release with PNGase F	LC-ESI-MS	Label free and labeling with isotope-coded protein label reagent	Monitorization of protein changes in the cell surface glycoproteome of *Drosophila melanogaster* cells.	[100]
Glycopeptide	Hydrazide resin	Human hepatocellular carcinoma cells	Cell culture supernatant collection / protein extraction / protein digestion (trypsin) / peptide oxidation / mixture with hydrazide resin / washing / glycopeptide release with PNGase F	nanoLC-ESI-MS	Label free	Analysis of *N*-glycosylation sites on secreted proteins of human hepatocellular carcinoma cells	[120]
Glycopeptide and glycoprotein	Hydrazide resin	Standard protein mixture (bovine fetuin, ribonuclease B, α1-acid glycoprotein, calf thymus histone type II-AS, bovine serum albumin, human IgG)	Glycopeptide level: individual protein digestion / peptide mixture / peptide oxidation / mixture with hydrazide resin / washing / glycopeptide release with PNGase F Glycoprotein level: protein oxidation / mixture with hydrazide resin / protein digestion (trypsin) / washing / glycopeptide release with PNGase F	LC-ESI-MS	Label free	Comparison of glycopeptide-enrichment strategies for quantitative site-specific analysis of protein glycosylation	[22]

Table 2. Continued

Glycoprotein/ glycopeptide level	Support	Sample	Sample treatment	LC-MS	Labeling	Objective	References
Glycoprotein	Hydrazide-modified super paramagnetic silica particles	Human α1-acid glycoprotein and mouse plasma	AGP ^{14}C radioactive labeling / ^{14}C-AGP spiking in mouse plasma / protein oxidation in 96-well plate / addition of hydrazide magnetic particles / washing / protein digestion (trypsin) / washing / glycopeptide release with PNGase F	LC-ESI-MS	^{14}C-labeling	Synthesis and evaluation of super paramagnetic silica particles with hydrazide groups on the surface for glycopeptide extraction	[112]
Glycopeptide	Hydrazide modified-Fischer type polymer	Model glycoproteins (α-fetoprotein, bovine pancreas fibrinogen, recombinant human erythropoietin)	Protein digestion (trypsin, chymotrypsin) / sialo-glycopeptide oxidation / addition of polymer reagent / polymer purification by column chromatography / sialoglycopeptide release by acid hydrolysis (TFA)	MALDI-MS	Label free	Method development for the selective enrichment of sialylated glycopeptides using the glycoblotting strategy.	[138]
Glycopeptide	Hydrazide modified-Fischer type polymer	Asialofetuin	Protein digestion (trypsin) / peptide oxidation (galactosyl oxidase) / addition of polymer reagent / washing / release by tansoximization or with PNGase F	MALDI-MS	Benzyloxyamine labeling and label free	Development of a one-pot solid-phase glycoblotting and transoximization procedure for glycomics and glycoproteomics	[137]
Glycoprotein	Hydrazide resin	Human plasma, tissue, culture breast cells, T and B cells	Protein extraction (tissue and culture breast cells) / protein oxidation / mixture with hydrazide resin / protein digestion (trypsin) / washing / glycopeptide release with PNGase F / strong cation exchange fractionation T and B cells: cell oxidation / cell labeling with biocytin hydrazide / cell lysis and membrane preparation / protein digestion (trypsin) / streptavidin glycopeptide capture / washing / glycopeptide release with PNGase F / strong cation exchange fractionation	LC-ESI-MS	Label free	Identification of *N*-linked glycosylation sites and glycoproteins from cultured cells, solid tissues, and plasma via glycopeptide capture and LC-MS analysis	[132]
Glycopeptide	Hydrazide resin	Biological samples	Protein digestion (trypsin) / peptide oxidation / mixture with hydrazide resin / washing / glycopeptide labeling with (d0/d4 succinic anhydride / glycopeptide release with PNGase F	LC-ESI-MS	Labeling with d0/d4 succinic anhydride	Protocol description for glycopeptide enrichment using hydrazide capture at the peptide level	[101]
Glycoprotein and glycopeptide	Hydrazide resin	Standard proteins (α1-antitrypsin, α1-acid glycoprotein) and mouse plasma	Glycoprotein level: standard proteins ^{14}C radioactive labeling / ^{14}C-proteins spiking in mouse plasma / protein oxidation / mixture with hydrazide resin / washing / protein digestion (trypsin) / washing / glycopeptide release with PNGase F / labeling with d0/d4 succinic anhydride Glycopeptide level: standard proteins ^{14}C radioactive labeling / ^{14}C-proteins spiking in mouse plasma / protein digestion (trypsin) / peptide oxidation / mixture with hydrazide resin / washing / glycopeptide release with PNGase F / labeling with d0/d4 succinic anhydride	ESI-MS	Labeling with d0/d4 succinic anhydride	Description and optimization of a protocol for blood plasma *N*-glycoprotein analysis using hydrazide capture. Comparison of capture at the glycoprotein or glycopeptide level	[93]

Table 2. Continued

Glycoprotein/ glycopeptide level	Support	Sample	Sample treatment	LC-MS	Labeling	Objective	References
Glycopeptide	Hydrazide resin	Chicken avidin, glycoprotein mixture, and an ovarian cell line	Microsomal fraction extraction / protein digestion (trypsin) / protein oxidation / mixture with hydrazide resin / washing / glycopeptide release with PNGase F	MALDI-MS and nanoLC-ESI-MS	Label free	Development of a shotgun glycoproteomics approach to comprehensively profile glycoproteins in complex biological mixtures	[96]
Glycoprotein	Hydrazide gel	Mouse plasma	Protein oxidation / mixture with hydrazide gel / washing / reduction + alkylation with biotin / protein digestion (trypsin) / washing / glycopeptide release with PNGase F / biotinilted cysteine containing de-glycopeptides capture with avidin / elution of cysteine containing glycopeptides	nanoLC-ESI-MS	Label free	Development of a strategy consisting of two orthogonal peptide affinity capture techniques for the isolation of cysteine-containing glycopeptides	[126]
Glycoprotein	Hydrazide resin	Human serum and plasma samples	Protein oxidation / mixture with hydrazide resin / washing / protein digestion (trypsin) / washing / glycopeptide release with PNGase F	nanoLC-ESI-MS	Label free	Development of a method based on the isolation of *N*-glycosites from plasma for their detection and quantification in the multiple reaction monitoring mode	[127]
Glycoprotein	Hydrazide gel	Human serum	Protein oxidation / mixture with the hydrazide gel / washing / protein digestion (trypsin) / washing / glycopeptide release with PNGase F	nanoLC-ESI-MS	Label free	Comparison of serum fractionation techniques (*N*-glycopeptide enrichment, cysteinyl-peptide enrichment, magnetic bead separation, size fractionation, protein A / G depletion, immunoaffinity depletion of abundant serum proteins)	[106]
Glycoprotein	Hydrazide gel	Human platelets	Human platelet purification / platelet lysis / protein oxidation / mixture with hydrazide gel / protein digestion (trypsin) / washing / glycopeptide release with PNGase F	nanoLC-ESI-MS	Label free	Elucidation of *N*-glycosylation sites on human platelet proteins	[54]
Glycoprotein	Hydrazide resin	Human saliva	Protein precipitation or IEF fractionation / protein oxidation / mixture with hydrazide resin / washing / protein digestion (trypsin) / washing / glycopeptide elution with PNGase F	LC-ESI-MS	Label free	Salivary glycoprotein identification	[128]

Table 2. Continued

Glycoprotein/ glycopeptide level	Support	Sample	Sample treatment	LC-MS	Labeling	Objective	References
Glycoprotein	Hydrazide resin	Cerebrospinal fluid	Protein oxidation / mixture with hydrazide resin / washing / protein digestion (trypsin) / washing / glycopeptide release with PNGase F / 2D LC (strong cation exchange + reverse phase) peptide fractionation	LC-ESI-MS	Label free	Glycoprotein identification in cerebrospinal fluid using hydrazide chemistry and lectin affinity for glycoprotein enrichment	[119]
Glycoprotein	Hydrazide resin	Human plasma samples	MIXED12 depletion / protein oxidation / mixture with hydrazide resin / washing / protein digestion (trypsin) / washing / glycopeptide release with PNGase F / SCX de-glycopeptide fractionation	LC-ESI-MS	Label free	Glycoprotein identification in trauma patient plasma using fractionation via hydrazide chemistry and via cysteinyl peptide capture	[122]
Glycopeptide	Hydrazide modified-Fischer type polymer	Synthetic glycopeptides	Peptide oxidation (galactosyl oxidase) / addition of polymer reagent / spinfiltration / sialoglycopeptide release by acid hydrolysis (TFA)	MALDI-MS	Label free	Development of glycoblotting for capturing of oligosaccharides and aldehyde-attached glycopeptides derived by enzymatic modifications	[136]
Glycoprotein	Hydrazide resin	Human plasma samples	MARS 6-depletion / protein oxidation / mixture with hydrazide resin / washing / protein digestion (trypsin) / washing / glycopeptide release with PNGase F / SCX de-glycopeptide fractionation	LC-ESI-MS	Label free	Analysis of the human plasma *N*-glycoproteome by hydrazide chemistry combined with high-abundance plasma proteins depletion	[105]
Glycoprotein	Hydrazide resin	Mouse serum samples (skin carcinoma and healthy mice)	Protein oxidation / mixture with hydrazide resin / washing / protein digestion (trypsin) / washing / glycopeptide labeling (d0/d4 succinic anhydride / glycopeptide release with PNGase F	LC-ESI-MS	Labeling with d0/d4 succinic anhydride	Method development for quantitative serum proteome analysis	[102]
Glycoprotein	Hydrazide resin	Human serum sample, LNCaP microsomal membrane fraction, and protein mixture (α-1-antitrypsin, α-2-hs glycoprotein, α-1-anti-chymotrypsin)	Protein oxidation / mixture with hydrazide resin / washing / protein digestion (trypsin) / washing / glycopeptide labeling (d0/d4 succinic anhydride / glycopeptide release with PNGase F	μLC-ESI-MS and MALDI-MS	Labeling with d0/d4 succinic anhydride	Development of the hydrazide capture method	[92]

CSC, cell surface capturing; SCX, strong cation exchange; TiO2, titanium dioxide.

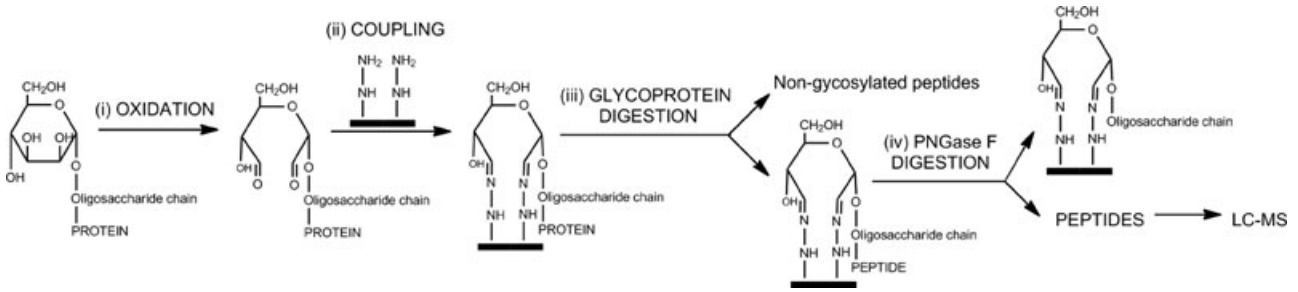

Figure 1. Hydrazide chemistry for glycopeptide enrichment.

oxidized proteins using biocytin hydrazide (a bifunctional linker molecule containing biotin), (iii) cell lysis and digestion of membrane proteins, (iv) streptavidin affinity enrichment of biocytin hydrazide-labeled peptides, and (v) deglycosylation with PNGase F. This method was able to identify cell surface proteins with less than 5% of identified proteins resulting from the coisolation of intracellular or nonglycosylated proteins [133]. Recently Hofmann et al. [135] have developed variants of the cell surface capturing technology that enabled the enrichment of glycopeptides as well as cysteine and lysine containing peptides from the surface of acute myeloid leukemia cells.

Based on a similar principle, Nishimura and coworkers developed a glycoblotting strategy for capturing carbohydrates and glycopeptides from complex samples [136]. This strategy is based on polymer reagents with reactive and stable aminooxy functional groups that can bind covalently to aldehydes allowing the trapping of oxidized (enzymatically [136, 137] or chemically [138]) oligosaccharides. These polymers allow trapping and purification of oligosaccharides or glycopeptides by spin filtration followed by their release through acid hydrolysis [136, 138], PNGase F, or transoximization [137]. Recently, this strategy was extended toward hydrazide-functionalized polymers [123].

3.2 Boronic acid chemistry

Boronic acid chemistry can be employed for the capture and isolation of cis-diol-containing molecules since boronic acids can form covalent bonds with 1–2 and 1–3 cis-diol groups to generate stable cyclic esters (see Fig. 2). Cyclic boronate esters are selectively formed at high pH and the reaction can be reversed under acidic conditions [139]. Thus, boronic acid chemistry can be employed for the enrichment of glycopeptides containing saccharides like mannose, galactose, or glucose. Contrary to lectins, the interaction between the glycopeptide and boronic acid does not require a complex recognition motif consisting of several saccharides. Thus, this strategy allows the capture of a broader range of *N*- and *O*-linked glycopeptides [140–142] as well as glycated peptides [143–146]. Since this method is based on covalent interactions, it allows to remove compounds adsorbed by noncovalent interaction to be washed away. Furthermore, the capture/release can be easily controlled through a simple switch of the pH and the acidic solutions required for elution are compatible with mass spectrometry. All this has made boronic acid matrices very attractive for the unbiased enrichment of both *N*- and *O*-glycopeptides (see Table 3).

Different materials such as monoliths [147], mesoporous silica [148], magnetic particles [24, 140], or gold nanoparticles-based materials [142, 149, 150] have been functionalized with boronic acid showing high specificity for glycopeptides. Among them, mesoporous and gold nanoparticles have high-surface areas that improve glycopeptide-binding rates as well as glycopeptide adsorption capacity [24, 142, 148–150].

One of the most widely used ionization techniques for the MS analysis of glycopeptides is matrix-assisted laser desorption ionization (MALDI). Commercial MALDI plates are generally made of stainless steel. However, grafting affinity probes onto MALDI plates for on-plate enrichment of target molecules presents several advantages when compared with conventional solid-phase extraction (SPE) methods since solution transfer and eluting steps are not needed, preventing sample loss and contamination. In this regard, on-plate glycopeptide enrichment strategies have been developed for direct MALDI-MS analysis [141, 151]. These strategies allow simplifying sample manipulation as well as reducing sample loading to amounts smaller than 5 μL. However, the reusability of these materials is limited and, although sample losses should be minimized, glycopeptide recovery is lower than for in-solution glycopeptide enrichment methods [151].

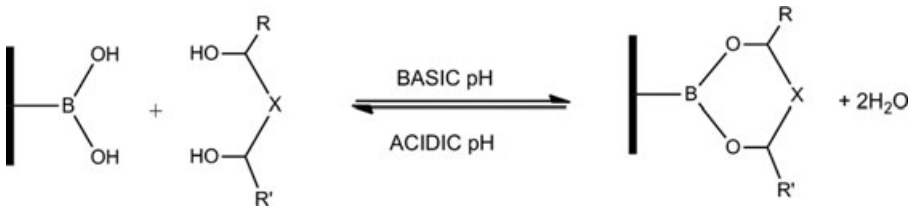

Figure 2. Boronic acid chemistry for covalent bond formation with 1–2 (X = none) and 1–3 (X = CH_2) cis-diol groups.

Table 3. Boronic acid chemistry for glycopeptide enrichment

Support	Sample	Sample treatment	LC-MS	Labeling	Objective	References
Boronate affinity column	Human plasma and erythrocytes (healthy controls and diabetic patients)	Protein extraction (erythrocytes)/(immunodepletion)/protein digestion (trypsin)/boronate affinity chromatography/strong cation exchange fractionation	LC-ESI-MS	Label free	Identification of glycated peptides in plasma and erythrocytes from control and diabetic subjects	[146]
Boronic acid-functionalized magnetic mesoporous silica nanocomposites	Standard glycoproteins (asialofetuin, horseradish peroxidase)	Protein digestion (trypsin)/mixture with boronic acid-functionalized magnetic mesoporous silica nanocomposites/washing/elution	MALDI-MS	Label free	Synthesis of boronic acid-functionalized magnetic mesoporous silica nanocomposites for glycopeptide enrichment	[24]
Boronic acid-functionalized gold-coated Si wafer	Standard proteins (bovine serum albumin, horseradish peroxidase, asialofetuin, fetuin)	Protein digestion (trypsin)/sample spotting on the wafer or immersion of one piece of the wafer in the sample (dependent on sample volume)/decanting + (washing)	MALDI-MS	Label free	On-plate glycopeptide enrichment for MALDI-MS analysis	[141]
Mercaptophenylboronic acid-functionalized core-shell structure Fe_3O_4@C@Au magnetic microspheres	Standard proteins (ribonuclease B, myoglobin, β-casein, horseradish peroxidase)	Protein digestion (trypsin)/mixture with magnetic microspheres/washing/elution	MALDI-MS	Label free	Synthesis of mercaptophenylboronic acid-functionalized core-shell structure Fe_3O_4@C@Au magnetic microspheres for glycopeptide and glycoprotein enrichment	[150]
Boronate affinity tips	Glycated ribonuclease B and human serum albumin, and human sera (healthy and diabetic patients)	Human serum albumin isolation/protein digestion/boronate affinity purification with boronate affinity tips	MALDI-MS μLC-MS	Label free	Development and application of boronate affinity tips for isolation of glycated human serum albumin from nondiabetic and diabetic patients serum	[143]
Boronate affinity monolith	Horseradish peroxidase	Protein digestion (trypsin)/polymer monolith microextraction	MALDI-MS	Label free	Development of a boronate affinity monolith for glycopeptide and glycoprotein enrichment	[147]
Boronic acid core-satellite composite nanoparticles	Horseradish peroxidase	Protein digestion (trypsin)/mixture with boronic acid core-satellite composite nanoparticle/washing/elution	MALDI-MS	Label free	Development of boronic acid core-satellite composite nanoparticles for glycopeptide and glycoprotein enrichment	[142]
4-Mercaptophenylboronic acid functionalized gold nanoparticles	Standard glycoproteins (asialofetuin, horseradish peroxidase) and skimmed milk	Protein digestion (trypsin)/mixture with 4-mercaptophenylboronic acid functionalized gold nanoparticles/washing/elution	MALDI-MS	Label free	Development of 4-mercaptophenylboronic acid functionalized gold nanoparticles for glycopeptide enrichment	[149]

Table 3. Continued

Support	Sample	Sample treatment	LC-MS	Labeling	Objective	References
Boronic acid-modified gold nanoparticles spotted on a stainless-steel plate	Standard proteins (horseradish peroxidase, β-casein) and drinking milk	Protein digestion (trypsin)/spotting of the digest on the gold-modified MALDI plate/washing/addition of desorption agent	MALDI-MS	Label free	Development of an on-plate glycopeptide enrichment strategy using boronic acid-modified gold nanoparticles for MALDI-MS analysis	[151]
Boronic acid functionalized mesoporous silica	Standard proteins (bovine serum albumin, horseradish peroxidase, asialofetuin, invertase, fetuin)	Protein digestion (trypsin)/mixture with the boronic acid functionalized mesoporous silica/washing/elution	MALDI-MS	Label free	Development of a boronic acid functionalized mesoporous silica material for glycopeptide enrichment	[148]
Boronate affinity column	Human plasma	Glycation of human plasma/protein digestion (trypsin, Arg-C, Lys-C)/boronate affinity chromatography	LC-ESI-MS	Label free	Method development for the enrichment and analysis of glycated peptides using boronate affinity chromatography and electron transfer dissociation	[144]
Aminophenylboronic acid-functionalized magnetic nanoparticles	Standard proteins (asialofetuin, horseradish peroxidase, ribonuclease B, bovine serum albumin, myoglobin)	Protein digestion (trypsin)/mixture with the aminophenylboronic acid-functionalized magnetic nanoparticles/washing/elution	MALDI-MS	Label free	Synthesis of aminophenylboronic acid-functionalized magnetic nanoparticles for glycopeptide and glycoprotein enrichment	[140]
Boronate affinity column	Glycated ribonuclease and human serum samples	Boronate affinity chromatography/protein digestion/boronate affinity chromatography	LC-ESI-MS	Label free	Enrichment and analysis of nonenzymatically glycated peptides using boronate affinity chromatography and electron transfer dissociation	[145]

Recently, Qian and coworkers developed a nanodevice based on macroporous silica foam materials functionalized with boronic acid and amine groups that allows to perform glycoprotein digestion, selective enrichment of glycopeptides, and purification of nonglycopeptides in an integrated manner [152].

Boronic acid-functionalized materials described above have shown high specificity for glycopeptide enrichment. However, they present problems when isolating glycopeptides from complex mixtures containing high amounts of nonglycosylated peptides as they may inhibit the glycopeptide-binding process [24, 148, 150, 151]. In addition, the reaction between boronic acid and 1–2 and 1–3 cis-diol groups is not specific to glycans and other compounds such as nucleotides [13] may be captured as well.

4 Chromatographic separation and solid-phase extraction methods

The enrichment and separation of glycopeptides on the basis of their physico-chemical properties is an alternative approach for the analysis of complex mixtures and glycosylation site assignment. Separation of glycopeptides poses an exceptional challenge due to their hydrophilicity and the similar physico-chemical properties due to the attached glycans. Progress in glycopeptide separation and enrichment is closely related to improvements in the development of HPLC stationary phases (Table 4).

4.1 Reversed-phase chromatography

The hydrophilic glycan moiety of glycopeptides renders their retention on conventional reversed-phase (RP) columns rather difficult. For example, the logarithm of the 1-octanol/water partition constant ($\log K_{ow}$) for a nonglycosylated transferrin-derived peptide CGLVPVLAENYN is reduced 2–3 times due to the attachment of one or two GlcNAc residues, respectively. The size of the glycan (number of attached saccharides) is the main determinant of the retention time of glycopeptides [153]. However, RP HPLC usually is not able to provide an appropriate separation of glycopeptides with similar size of the glycan moiety (but different glycan composition) due to their similar hydrophobicity [154]. To obtain additional information and detect site-specific glycoforms of glycopeptides, Medzihradszky et al. employed a mobile phase consisting of water/ethanol/propanol/formic acid, which provided a different separation selectivity in comparison with water/acetonitrile/formic acid [155]. Selective glycopeptide mapping of human erythropoietin was achieved by separation of glycopeptides on an ODS column with a mobile phase containing 1 mM ammonium acetate at pH 6.8 and two-step gradient of acetonitrile [156]. Separation was based on the number of sialic acid and *N*-acetyllactosaminyl repeats as well as on differences branching. The method was applied for the identification of glycosylation sites in endoproteinase Glu-C-digested erythropoietins from different sources [157].

Table 4. Stationary phases for glycopeptide separation and enrichment

Name and details	Manufacturer	References
Cation exchange materials		
PolySULFOETHYL Aspartamide, 2.1 × 150 mm, 200-Å pore size, 5 μm	Chromatographic Technologies, Basel, Switzerland	[158]
PolySULFOETHYL A 4.6 × 200 mm 200-Å pore size, 5 μm	PolyLC, Columbia, MD, USA	[238]
Weak cation-exchange/crown ether column, IonPac CS 15 of 2 × 250 mm, 8 μm particulate resin of 55% crosslinked ethylvinylbenzene/divinylbenzene, functionalized with phosphonate, carboxylate, and crown ether groups.	Dionex, Amsterdam, The Netherlands	[160]
Gel filtration materials		
Bio-Gel P-30 column, 100–200 mesh; 1.6 × 160 cm	BioRad, Munich, Germany	[154]
Size-exclusion chromatography		
Superdex Peptide 10/300 GL column, 2.1 mm × 250 mm	GE Healthcare Bio-Sciences	[162]
Carbon and graphite materials		
Carbograph (nonporous graphitized carbon)	Shandon Scientific, Cheshire, UK	[163]
Hypercarb (porous graphitized carbon), 4.6 × 10 mm, 70 μm		
Hypercarb, 2.1 × 100 mm, 5 μm	Hypersil, UK	[172, 173]
Porous graphitized carbon solid-phase extraction cartridge	Alltech Associate inc., Deerfield, IL, USA	[165, 174]
Microfluidic chip consisted of 75 μm × 9 mm enrichment column and 75 μm × 150 mm analytical column packed with 5 μm porous graphitized carbon	Grace Davison Discovery Sciences, Deerfield, IL, USA	[166, 168]
LC chips containing 40-nL trapping column and a 75 μm × 43 mm separation column packed with activated graphitized carbon	Agilent Technologies, Inc., Palo Alto, CA, USA	[167]
Graphite powder packed in GELoader tips	Catalog number C5510, Sigma	[169]
Graphite microspin columns	Harvard Apparatus, Holliston, MA	[170]
Graphite carbon powder	Alltech, Sydney, Australia	[171]
Polyhydroxyethyl Aspartamide materials		
Polyhydroxyethyl Aspartamide, 1 × 150 mm	PolyLC, Columbia, MD, USA	[188]
Polyhydroxyethyl ATM Javelin® guard column, 1 × 10 mm, 5 μm	PolyLC, Columbia, MD, USA	[189]
Zwitterionic materials		
ZIC®-HILIC chromatography resin, 10 μm, packed in tips (Eppendorf, Hamburg, Germany)	Sequant, Umeå, Sweden	[49, 190–193] [22, 195–197]
ZIC®-HILIC, 2.1 × 150 mm, 3.5 μm	Sequant, Umeå, Sweden	[198]
ZIC®-HILIC, 2.1 × 150 mm, 3.5 μm, 20 nm	Sequant, Umeå, Sweden	[199]
ZIC-HILIC-modified monolithic silica capillary	Merck SeQuant AB, Sweden	[200]
ZIC®-HILIC, 200 Å 2.1 × 50 mm, 3.5 μm	Sequant, Umeå, Sweden	[201]
ZIC®-HILIC column, 0.075 × 150 mm, 3.5 μm	Sequant, Umeå, Sweden	[202]
Click TE-Cys column (zwitterionic stationary phase synthesized based on the "thiol-ene" click reaction between cysteine and vinyl silica), 4.6 × 150 mm	Key Lab of Separation Science for Analytical Chemistry, Dalian, China	[203]
ZIC®-HILIC guard column, 1 × 5 mm, 5 μm	Nest Group, Southborough, MA, USA	[189]
Amide materials		
Nanoscale Amide-80 column, 75 μm × 100 mm, 5 μm; 80 Å	Tosohaas, Montgomeryville, PA, USA	[204]
Nanoscale Amide-80 column, 75 μm × 180 mm, 3 μm; 80 Å	Tosoh Bioscience, Stuttgart, Germany	[205]
TSKgel Amide 80, 5 μm, 300°A	Tosoh Bioscience, Montgomeryville, PA, USA	[206]
TSK guard gel Amide-80, 2.0 × 10 mm, 5 μm	Tosoh Bioscience GmbH, Stuttgart, Germany	[22]
ACQUITY Bridge-ethyl hybrid silica (BEH) glycan column, 2.1 × 150 mm, 1.7 μm	Waters, Milford, MA, USA	[207]
Micro-capillary column 320 μm × 170 mm packed with TSK Amide-80 3 μm	Tosoh Bioscience, San Francisco, CA, USA	[97]
Amine materials		
Aminopropyl silica sorbent	Waters	[208, 209]
Microsorb 100–10 Amino	Varian Inc., Palo Alto, CA, USA	[22]
Luna NH_2 column, 0.3 × 10 mm, 5 μm	Bodman Industries, Aston, PA, USA	[189]

Table 4. Continued

Name and details	Manufacturer	References
Cellulose, sepharose, and cotton materials		
Sepharose CL-4B	Amersham Bioscience (Piscataway, NJ, USA	[211]
Sepharose CL-4B	Merck, Darmstadt, Germany	[210]
Cellulose microcrystalline	Amersham Bioscience, Piscataway, NJ, USA	[210]
Sepharose CL-6B	Sigma-Aldrich, St. Louis, MO, USA	[232]
Sepharose 4B	Amersham Biosiciences, NJ, USA	[214]
Cellulose Cartridge Column Pack	TaKaRa, Kyoto, Japan	[214]
Sepharose CL-4B beads, 45–165 μm	GE Healthcare, Uppsala, Sweden	[215]
Cellulose microcrystalline particles for column chromatography, 5 mg	Merck KGaA, Germany	[215, 216]
Cotton wool packed in tips	Da, Dynaretail, Leusden, The Netherlands; Etos, Etos bv, Beverwijk, The Netherlands; Bella, Groupe Lemoine; Paris, France	[217]
Sepharose CL-6B	Sigma, St. Louis, MO, USA	[194]
Saccharide-based materials		
Click chitooligosaccharide, 2.1 × 100 mm, 5 μm	Key Lab of Separation Science for Analytical Chemistry, Dalian, China	[218]
Click glycosyl phenyl glycine, 2.1 × 100 mm, 5 μm	Key Lab of Separation Science for Analytical Chemistry, Dalian, China	[218, 219]
Click maltose packed in tips	Key Lab of Separation Science for Analytical Chemistry, Dalian, China	[220, 221]
Click maltose column, 300 μm × 50 mm	Key Lab of Separation Science for Analytical Chemistry, Dalian, China	[222]
Click OEG-CD (β-cyclodextrin linked onto the surface of silica gel through an oligo (ethylene glycol) (OEG) spacer via click chemistry	Key Lab of Separation Science for Analytical Chemistry, Dalian, China	[223]
TiO_2 and ZrO_2 materials		
Titanium dioxide beads were obtained from a disassembled TiO_2 column, titansphere, 4.6 × 250 mm, 5 μm	GL Sciences Inc., Tokyo, Japan	[225]
Titanium dioxide microspheres synthesized by the sol–gel method	Key Lab of Separation Science for Analytical Chemistry, Dalian, China	[226]
Titanium dioxide beads	GL Sciences, Tokyo, Japan	[97, 228–231]
Zirconia-coated mesoporous silica (ZrO_2/MPS) microspheres	Key Lab of Separation Science for Analytical Chemistry, Dalian, China	[232]
MagPrep TiO_2	Merck KGaA, Germany	[22]
Weak-anion exchange materials		
PolyWAX LPTM column 4.6 × 100 mm, 5 μm, 300 Å	Columbia, MD, USA	[234]
PolyWAX LPTM column 4.6 × 200 mm, 5 μm, 300 Å	Columbia, MD, USA	[129, 236, 238]

Click OEG-CD, click β-cyclodextrin oligo (ethylene glycol); ZIC-HILIC, zwitterionic HILIC.

4.2 Cation-exchange chromatography

Lewandrowski et al. used a polysulfoethyl aspartamide (Fig. 3) column to separate glycopeptides with attached sialic acids [158] from nonglycosylated peptides and peptides with neutral glycan moieties. The presence of additional negatively charged groups resulted in elution of sialic acid contained glycopeptides in the flow-through fraction with little contamination by other peptides allowing to identify 148 glycosylation sites on 79 platelet membrane proteins after strong cation exchange (SCX) prefractionation [158]. It should be noted that SCX separation was performed at pH 2.7, which corresponds to the pK_a value of *N*-acetyl neuraminic acid [159]. In addition, a weak cation exchange/crown ether column, originally proposed for the analysis of inorganic ions was employed for the separation of peptides with free α-NH_2 or ε-NH_2 groups from lysine side chains by Tuytten et al. [160]. Interestingly, this also led to the co-elution of glycopeptides [160]. Unfortunately, the investigation of this interesting column for proteomics purposes was not continued and the mechanism of separation remains unclear.

4.3 Size-exclusion chromatography (SEC)

According to Alvarez-Manilla et al., glycopeptides comprise only a small part (2–5%) in the peptide mixtures compared to nonglycosylated peptides in tryptic digested peptide

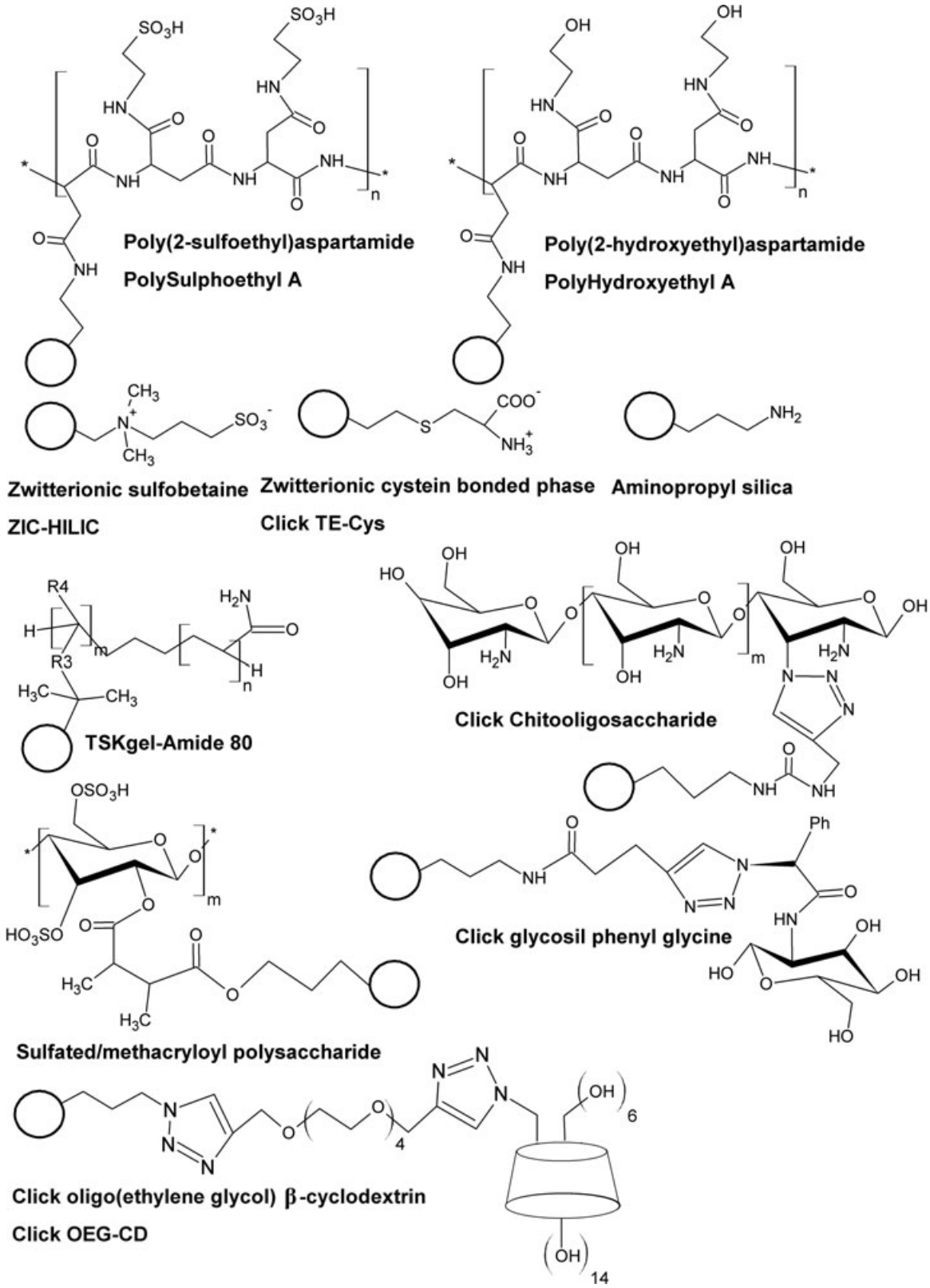

Figure 3. Structures of some HILIC stationary phases.

mixtures [161]. Based on the significant contribution of *N*-linked glycans to the size of tryptic glycopeptides, the possibility to separate them from nonglycosylated peptides on a Superdex Peptide column from complex protein mixtures using size exclusion chromatography was demonstrated [161]. In total, three times more *N*-linked glycopeptides were identified by LC-MS/MS of a tryptic digest of human serum after SEC prefractionation followed by a PNGase-F treatment and were identified by monoisotopic mass increase of 0.984Da for asparagine compared to analysis of peptide mixture that was not subjected to SEC [161]. The same approach was used for glycopeptides that were separated from a depleted plasma (from six most abundant proteins) fraction and serotransferrin digests by SEC on a Superdex Peptide column [162].

4.4 Porous graphitized carbon (PGC) chromatography

Packer and coworkers [163] demonstrated that graphitized carbon can be successfully used for the purification of oligosaccharides from solutions with salts, monosaccharides, proteins (including enzymes), and other components. Complete recovery of *N*-linked oligosaccharides from a graphitized carbon cartridge of PNGase-treated urine samples was demonstrated prior to mass spectrometry [163]. SPE on graphitized carbon cartridges was implemented in a study of glycopeptides in human plasma [164]. The graphitized carbon cartridges used for SPE followed by high-mass accuracy and high-resolution mass spectrometry resulted only in glycopeptide mass spectra. The usefulness of this method was shown for single glycoproteins analysis and not for complex protein mixtures [165]. A comparison of graphitized carbon chips (9 × 0.075 mm and 150 × 0.075 mm id for enrichment and analytical columns, respectively; both contained porous graphitized carbon) with C8 or C18 stationary phase chips for the enrichment/separation of hydrophilic glycopeptides showed that both *N*- and *O*-glycopeptides (in total 18 glycosites) were detected in the protein mixture after Pronase E (bound to Sepharose beads) digestion using PGC chromatography [166]. However, identification of hydrophilic and hydrophobic glycopeptides was best achieved by combining activated graphitized carbon and C18 chips [167]. Using microfluidic graphitized porous carbon chips allowed to enrich isomers of glycopeptides with confirmation of glycopeptide composition and resulted in information about the glycan and peptide structures (59 glycans at five *N*-glycosylation sites on bovine lactoferrin) [168]. Prefractionation of glycoproteins by 2-D gel electrophoresis, in-gel digestion complemented by desalting and concentration of the glycopeptides on nl-volume microcolumns packed with graphite powder followed by MALDI-MS analysis allowed to detect glycopeptide signals above m/z 1200 [169]. Graphite micro-spin columns were employed during sample preparation of serum (5 μL) in a study related to ovarian cancer [170]. A microcolumn in a pipette tip format containing a mixture of graphitized carbon and activated charcoal (w/w, 1:1 ratio) allowed the enrichment of *N*-glycopeptides of a few glycoproteins after digestion [171].

Kawasaki et al. showed that PGC chromatography allowed the separation of diverse oligosaccharides and oligosaccharide alditols released from a glycoprotein [172, 173] and determination of their relative abundance [174]. The wide use of PGC in glycan and glycopeptide analysis can be explained by the robustness of the material (no change in performance over long time periods) and the possibility to separate isomers [175].

4.5 Hydrophilic-interaction chromatography

In 1990, Alpert [176] introduced the term HILIC for the normal-phase chromatographic mode with a polar stationary phase and a less polar mobile phase. However, in contrast to classical normal-phase chromatography, which uses water-nonmiscible mobile phases, the mobile phase in HILIC comprises a mixture of a water-miscible organic solvent (usually acetonitrile) with a certain amount of water (45% or less). The popularity of HILIC is related to the opportunity to separate polar compounds, compatibility of mobile phases based on acetonitrile, and a volatile pH adjusting reagents with mass spectrometric detectors and its orthogonality to RP HPLC [176–178]. The mechanism of HILIC with respect to different stationary phases [179–182], efficiency [183], retention and selectivity [184, 185] is still under thorough investigation [186]. However, the diverse nature of available HILIC stationary phases makes the practical application of the observed principals to different compound classes difficult [187] and optimal separation conditions are usually selected based on trial and error.

4.5.1 Polyhydroxyethyl aspartamide HILIC

The first application of HILIC to glycopeptide separation was published by Zhang and Zhang in 1998 [188]. A 2D LC with an RP separation in the first dimension and HILIC in the second dimension followed by MALDI-TOF-MS allowed to characterize the site-specific glycosylation microheterogeneity of interferon-γ mainly due to the differences in retention of peptides with different numbers of the sialic acid residues in their oligosaccharide structures in the HILIC dimension [188]. Ding et al. [189] compared ZIC®-HILIC, Luna NH_2, and polyhydroxyethyl aspartamide (Fig. 3) columns (see Table 4) for glycopeptide separation and concluded that polyhydroxyethyl aspartamide provided the best separation. An interesting observation was the profound effect of the acid in the mobile phase on the quality of separation; glycosylated peptides were well separated with additives such as hydrochloric or trifluoroacetic acid but co-eluted if acetic acid was used for pH regulation [189].

4.5.2 Zwitterionic HILIC (ZIC-HILIC)

Hagglund et al. showed improvements in *N*-glycosylation site identification after capture of glycosylated peptides on a stationary phase with bound zwitterionic sulfobetaine functional groups in the HILIC mode [190]. The combination of lectin affinity enrichment of proteins with 1D gel electrophoresis, in-gel digestion, and HILIC allowed to detect 62 glycosylation sites in 37 proteins from human plasma [190]. ZIC-HILIC (Fig. 3) microcolumns for SPE [190] were used for the enrichment of glycosylphosphatidylinositol-containing peptides from porcine kidney membrane dipeptidase [191]. The analysis of this complex posttranslational modification, i.e. covalently linked to the C-terminus of the protein is a demanding analytical task and the considerable enrichment of the corresponding peptide with ZIC-HILIC provided further insight into its structure. Approximately, the same number of glycopeptides and proteins were identified in serum after glycopeptides were enriched by ZIC-HILIC or a mixture of immobilized lectins [49]. Partial removal of more hydrophobic nonglycosylated peptides by reversed-phase microcolumns and subsequent enrichment of glycopeptides with a ZIC-HILIC microcolumn allowed characterization of the glycosylation sites of human β_2-glycoprotein I after thorough optimization of the respective resin volumes, which proved to be essential for reproducible recovery of glycopeptides [192]. *N*-glycosylation sites of tissue inhibitor of metalloproteinases-1 (TIMP-1), a low-abundant (50–80 ng/mL) protein in plasma from five healthy individuals were determined using a similar two-step glycopeptide enrichment method [193]. Contamination of glycopeptides with nonglycosylated hydrophilic peptides after SPE on ZIC-HILC can be reduced by addition of trifluoroacetic acid as an ion-pair reagent (see also [189, 194]) [195]. It should be noted that the identified glycopeptides were uniformly distributed among the complete set of known glycopeptides indicating an unbiased enrichment by ZIC-HILIC. Picariello et al. [196] packed ZIC-HILIC into pipette tips for enrichment of glycopeptides after tryptic digestion of human milk proteins. The procedure was not fully specific as a number of nonglycosylated peptides, mainly very hydrophilic peptides (e.g. contained three contiguous acidic amino acids such as EDE, or EEE) were nonspecifically retained on the HILIC resin. Despite this fact, the enrichment of glycopeptides from a complex *Campylobacter jejuni* peptide mixture with ZIC-HILIC allowed to determine novel glycosylation sites using a combination of collision-induced dissociation (CID), higher energy CID, and CID/electron transfer dissociation (ETD) MS [197]. The simultaneous implementation of CID/higher energy CID and CID/ETD allowed to overcome signal disruption caused by decreased ETD efficiency for peptides with m/z values higher than 850 and provided peptide sequence and glycosylation site information. Wohlgemuth et al. [22] conducted a comprehensive comparison of *N*-glycopeptide enrichment with different ZIC-HILIC materials and hydrazide chemistry (see Section 2.1).

The application of ZIC-HILIC for the separation of *N*-glycopeptides and 2-aminopyridine derivatized glycans of human IgG (immunoglobulin G) showed new prospects for glycoprotein analysis [198]. The advantages of ZIC-HILIC include: (i) the separation of *N*-glycopeptides from nonglycosylated peptides; (ii) the separation of *N*-glycopeptide isomers for further ESI-MS analysis (e.g. glycopeptides with different positions of galactose) [198]. The separation of *N*-glycopeptides of an α_1-acid glycoprotein digest on the same ZIC-HILIC column revealed additional features: (i) sialylated *N*-glycopeptides are separated from neutral glycans; (ii) sialylated *N*-glycopeptides with relatively large isomeric tri- and tetra-antennary *N*-glycan structures are well separated based on the structural recognition for different linkage types [199]. Even better separation of glycopeptides was obtained by utilizing ZIC-HILIC and monolithic RP columns sequentially since ZIC-HILIC resolves different glycan structures while RP provides the efficient separation of glycopeptides based on their peptide sequence and degree of sialylation [200]. Such a strategy was, however, only applied to the analysis of digested pure proteins [22, 200]. Optimization of the mobile phase pH by changing the concentration of formic acid significantly improved the separation of *O*-sialoglycopeptides from a trypsin/chymotrypsin digest of caseinomacropeptide on a ZIC-HILIC column [201]. The electrostatic repulsion between the localized negative charge on the sialylated oligosaccharides and that on the stationary phase is most probably responsible for the changing selectivity at different pH values of the mobile phase. The best separation was observed under conditions where the negatively charged, dissociated sialic acid predominates in solution (0.005% (v/v) formic acid, pH between 3.2 and 3.6) [201]. The capability to separate simultaneously nonglycosylated peptides and *N*- and *O*-glycopeptides with ZIC-HILIC was demonstrated by Takegawa et al. [202]. The predictable elution of *O*-glycopeptides with smaller saccharide moieties at intermediate retention times (20–30 min) and of *N*-glycopeptides with large (tri- and tetraantennary complex-type oligosaccharides) glycans later in the gradient was observed [202]. A novel zwitterionic HILIC stationary phase based on "thiol-ene" click chemistry between cysteine and vinyl silica (Click TE-Cys, Fig. 3) was recently synthesized by Shen et al. particularly for glycopeptide separation [203]. The independent comparison of this material with commercial ZIC-HILIC must be completed to elucidate whether the Click TE-Cys stationary phase provides advantages over ZIC-HILIC.

4.5.3 Amide HILIC

Wuhrer et al. [204] proposed an approach comprising the unspecific proteolysis of proteins with Pronase and nano-scale LC-MS on an Amide-80 (Fig. 3) column for characterization of site-specific glycosylation. The advantage of the method is the formation of small (2–8 amino acid) glycosylated and nonglycosylated peptides after digestion, which were easily separated into two groups and separation of different glycoforms of the same peptide moiety due to increasing retention with increasing glycan size. The characterization of *N*- and *O*-glycosylation sites of asialofetuin and fetuin in a single

run was shown by Zauner et al. by nanoLC separation of Proteinase K generated peptides [205]. As for ZIC-HILIC materials, the retention of glycopeptides on Amide-80 was governed mainly by glycan size. The simultaneous identification of proteins based on nonglycosylated hydrophobic peptides and characterization of glycosylation sites can be achieved by online integration of an RP and an Amide 80 column [206]. Recently, a similar 2-D chromatographic approach, where RP chromatography was employed for the isolation of glycopeptides according to their amino acid sequence (hydrophobicity) and HILIC for resolution of the isolated glycopeptides into their glycoforms was used for glycosylation site identification of bovine fetuin, human α_1-acid glycoprotein, human transferrin, and bovine ribonuclease B (RNase B) [207].

4.5.4 Aminopropyl silica HILIC

The 96-well HILIC micro-elution plate packed with 5 mg of aminopropyl silica sorbent (Fig. 3) in each well was tested by Yu et al. for enrichment of glycans by SPE [208]. This approach was later applied for the enrichment of glycopeptides and the identification of *N*-linked glycosylation sites of RNase B, α_1-acid glycoprotein, horseradish peroxidase, and α_2-(3,6,8,9)-neuraminidase [209].

4.5.5 Cellulose, sepharose, and cotton-based HILIC

The idea to implement well-known carbohydrate materials for the enrichment of glycopeptides received much attention during the past two years. Wada et al. developed a simple method for glycopeptide enrichment which consisted of mixing the digested sample with carbohydrate-based gel matrices (cellulose or Sepharose) in an organic solvent followed by the extraction of captured glycopeptides under aqueous conditions [210]. The method was successfully applied for profiling the glycosylation sites of IgGs [210], fibronectines [211], transferrin and haptoglobin [212] and immunoglobulin A1 [213]. Addition of divalent cations (Mn(II), Mg(II), Ca(II), Ba(II), Co(II), and Ni(II)) was later proposed for improving glycopeptide recovery from Sepharose CL-4B [211]. The mechanism of metal ion action may be due to the formation of complexes with saccharides, but this requires further investigations. The protocol for glycopeptide enrichment with cellulose or Sepharose 4B has been described in detail by Ito et al. [214]. The authors proposed to apply HILIC enrichment of glycopeptides from peptides mixtures obtained after lectin affinity chromatography. Thus, the additional step of samples cleaning from the sugar must be included before the following hydrophilic extraction. The profiling of IgG glycosylation was performed by Selman et al. [215] with HILIC SPE of glycopeptides on cellulose microcrystalline particles or Sepharose CL-4B beads in a 96-well filter plate [215]. Although similar *N*-glycopeptide profiles were obtained with cellulose and Sepharose SPE, the latter is preferable due to a more homogeneous particle size [215]. It is interesting that the lower relative intensities observed for sialylated glycopeptides after HILIC SPE in comparison to RP SPE were caused by mass spectrometric signal suppression and could be eliminated by an extra washing step with ACN containing 0.1% formic acid [215]. The developed approach was successfully applied to study IgG glycosylation in relation to gender and age in humans showing, e.g. that sialylation decreases with age and that galactosylation is highest at age 25 while decreasing with increasing age [216]. Recently, Selman et al. [217] proposed to use commercially available cotton wool pads for desalting and enrichment of *N*-glycopeptides and tested the procedure on an IgG tryptic digest. The observed results were in agreement with previously reported data using SPE on Sepharose and microcrystalline cellulose [215, 217].

Sepharose CL-6B has also been used for glycopeptide separation in the column mode [194]. The observed improvement in selectivity for glycosylated and nonglycosylated peptide separation after addition of monovalent cations (NaCl, LiCl, and NaOH) may be related to a decrease in the effective peptide charge that resulted in increased hydrophobicity.

4.5.6 Saccharide-based HILIC

Novel hydrophilic saccharide-based materials synthesized by "click chemistry" have recently been tested for the enrichment of glycosylated peptides. Azidochitoologosaccharide (Fig. 3) attached to alkyne silica showed comparable results to Sepharose CL-6B for the enrichment of glycopeptides [218]. Enrichment was improved when the attached glycan moiety contained also an amino acid residue presumably due to more specific interactions between the glycosyl phenyl glycine moieties (Fig. 3) and the glycopeptides [219]. Unlike the rigid structure of Sepharose, the flexibility of click-maltose chains attached to silica beads provides a sufficient number of hydroxyl groups for the effective formation of hydrogen bonds that results in improved selectivity especially for nonglycosylated peptides, containing multiple serine or threonine residues, which may co-elute with glycopeptides [220]. It was recently shown that the same click maltose material is useful for desalting both neutral and sialylated *N*-linked glycopeptides providing a better recovery than RP sorbents [221]. An integrated sample pretreatment system, in which glycopeptides were first enriched with click-maltose, followed by a buffer exchange on an SCX precolumn and finally deglycosylation in an immobilized PNGase F reactor, allowed to achieve a 5 fmol detection limit for glycopeptides from a digest of avidin in the presence of a 50-fold excess of bovine serum albumin [222]. The combination of RP LC with SPE by using a click β-cyclodextrin oligo (ethylene glycol) (Click OEG-CD) matrix showed high selectivity for the enrichment of glycopeptides with multiple glycosylation sites [223]. Moreover, selectivity for glycopeptide enrichment of Click OEG-CD (Fig. 3) was different from that of click maltose in the HILIC mode and may give complementary information for the analysis of glycosylation sites. However, a comprehensive comparison of novel saccharide based and the more widely employed ZIC-HILIC and Amide-80 materials is needed to draw final conclusions on the effectiveness of

saccharide-based materials for glycopeptide enrichment from complex samples.

4.5.7 Titanium oxide HILIC

Titanium dioxide (TiO_2) was originally proposed for phosphopeptide enrichment from proteolytic digests [224] can also be used for glycopeptide enrichment. Enrichment of sialic acid-containing glycopeptides on TiO_2 microcolumns followed by mass spectrometry allowed characterizing the human plasma and saliva sialiomes (192 and 97 glycosylation sites were identified, respectively) [225]. Wohlgemuth et al. [22] captured sialic acid-containing glycopeptides with MagPrep TiO_2 from a mixture of digested bovine fetuin, RNase B, α_1-acid glycoprotein, and calf thymus histone type II-AS (a mixture of all histone subtypes) [225]. Titanium dioxide microspheres synthesized by the sol–gel method showed higher hydrophilicity than other HILIC materials such as Sepharose or click-maltose for the enrichment of neutral glycopeptides [226]. The idea to combine the removal of hydrophobic nonglycosylated peptides with an ODS sorbent followed by the enrichment of glycopeptides in the HILIC mode on TiO_2 (see also [192, 206, 227]) in one procedure was realized by Zhang et al. [228]. The majority of nonglycosylated peptides were thus removed enhancing the signals of glycopeptides, but some contaminating peptides with several serine or threonine residues were found during ESI-MS analysis [228]. An interesting approach for the indirect enrichment of *O*-GlcNAc-modified peptides was proposed by Parker et al. [229]. First, *O*-glycopeptides were enzymatically labeled with *N*-azidoacetylgalactosamine and the azide was subsequently reacted with a phospho-alkyne using click chemistry. Labeled *O*-glycosylated peptides were enriched using TiO_2 [229]. The procedure included enrichment of sialylated glycopeptides with TiO_2, deglycosylation, and fractionation of nonglycosylated peptides on a HILIC microHPLC column (Amide-80) as described by Palmisano et al. [230, 231]. The selectivity of TiO_2 toward sialic acid-containing glycopeptides was improved by using a low-pH buffer (5% (v/v) trifluoroacetic acid) that contained a substituted carboxylic acid such as glycolic acid. Recently, zirconia-coated mesoporous silica microspheres were proposed for SPE of glycopeptides and tested on a trypsin digest of IgG, RNase B, and α-casein resulting in better selectivity for glycopeptide enrichment and higher coverage of glycosylation sites as compared to enrichment on Sepharose or silica microspheres [232]. Recently, Parker et al. described an approach, which combined *N*-linked glycopeptide capture onto a hydrazide support, enrichment of sialic acid-containing *N*-linked glycopeptides with TiO_2, separation of *N*-linked glycopeptides using a ZIC-HILIC microcolumn and Amide-80 HILIC peptide fractionation for the identification of glycosylation sites and quantitative *N*-linked glycoproteomics of rat myocardial tissue membranes [97]. One thousand five hundred and fifty six *N*-linked glycosylation sites from the rat left ventricular myocardium were identified after induction of necrosis in the isolated organ [97].

4.5.8 Weak-anion exchange HILIC (electrostatic repulsion hydrophilic interaction chromatography, ERLIC)

The usefulness of weak-anion exchange materials in the HILIC mode for the analysis of protein posttranslational modifications was first demonstrated for phosphorylated peptides [233]. The opportunity to separate glycosylated from nonglycosylated peptides with polyWAX in the HILIC mode was subsequently shown by Lewandrowski et al. [234]. However, authors did not achieve the separation of different glycopeptide isoforms [234]. A discussion of HILIC and ERLIC performance for the characterization of posttranslational modifications suggested elimination of interfering phosphopeptides by phosphatase treatment [235]. The versatility of polyWAX in the HILIC mode for glyco- and phosphopeptide separation resulted in an approach where glycosylation and phosphorylation sites were determined in a single run after ERLIC prefractionation [129]. Glycopeptides were eluted across a broader gradient window (70–30% (v/v) acetonitrile) as compared to phosphopeptides (70–60%), which is probably due to the greater complexity and heterogeneity of the carbohydrate moieties [129]. The ERLIC approach was shown to be more efficient in the identification of glycopeptides and glycoproteins than the hydrazide chemistry method (3% of all identified glycoproteins were found with the hydrazine chemistry method and 66% with ERLIC) [129]. Recently, Hao et al. optimized the previously described ERLIC method for shotgun proteomics [236] for the separation of glyco and phosphopeptides from rat kidney tissue [238]. The comparison of ERLIC and SCX prefractionation of peptides showed better selectivity in the former case: the phospho- and glycopeptides were distributed over more than 40 ERLIC fractions after the elution of nonmodified peptides while co-elution of glyco and phosphopeptides in flow-through fractions was observed for the SCX separation [238]. As a result, the number of identified glyco and phosphoproteins was around 40% higher for the ERLIC method in comparison to SCX.

5 Glycopeptide separation: a methods comparison

Different authors have compared lectin affinity enrichment with other glycopeptide enrichment strategies such as hydrazide chemistry or HILIC. Takeshi et al. [88] found that the technique using hydrazide capture was more effective than the use of Con A and wheat germ agglutinin isolation kits for the enrichment of glycopeptides from human plasma since it allowed the enrichment and identification of a larger number of *N*-glycopeptides. However, Lewandrowski et al. [54] reported that Con A affinity enrichment and the hydrazide chemistry approach are complementary. Although some overlap was observed, many of the glycosylation sites were identified either by Con A or hydrazide chemistry but not both. Calvano et al. [49] compared four enrichment

strategies for the characterization of serum glycoproteins by mass spectrometry. The first strategy was based on glycopeptide enrichment with a mixture of lectins (wheat germ agglutinin, Con A, and *S. nigra* agglutinin), the second was based on HILIC glycopeptide enrichment, the third and fourth consisted of two enrichment steps; one at the glycoprotein level (multilectin affinity) and the second at the glycopeptide level (multilectin affinity in the third and HILIC in the fourth strategy). The two-step strategies proved to be more efficient. This group also showed that the application of a mixture of lectins is efficient, reproducible, and robust for the enrichment of intact glycoproteins from serum and subsequently also for their tryptic glycopeptides. The combination of a lectin mixture for glycoprotein enrichment followed by HILIC was also efficient, but slightly less reproducible. Zhang et al. [73] compared different chromatographic and/or enrichment approaches (RP-HPLC, HILIC, and lectin affinity) for glycopeptide analysis based on the number of detected glycosylation sites and identified glycopeptides in three model glycoproteins. The approach leading to the best coverage was RP-HPLC. When HILIC and lectin affinity enrichment methods were compared, lectin affinity showed the poorest reproducibility. When compared to other enrichment strategies, hydrazide chemistry has proven to be complementary to HILIC, lectin, or TiO_2 enrichment [54,108,115,118–120] although HILIC produced a higher glycopeptide identification when compared to the hydrazide capture [22, 97, 120, 129]. However, HILIC and lectin enrichment proved to be less specific than hydrazide-based enrichment [120]. Thaysen-Andersen et al. [238] compared the results of glycopeptide separation using HILIC columns packed with Amide-80 or ZIC-HILIC and enrichment of glycopeptides with other HILIC materials (ZIC-HILIC, PolyHydroxyethyl A, PolySulfethyl A, TSK Amide-80, and LudgerClean S), graphitized carbon (Hypersil and LudgerClean EB10), lectin affinity chromatography (ConA), and TiO_2 with the conclusion that none of the approaches gives a comprehensive glycoprofile and to that the capacity of the sorbent must be taken into account. The performance of SCX and ERLIC prefractionation of peptides for the simultaneous characterization of glycosylation and phosphorylation sites in complex protein mixtures was analyzed by Hao et al. [237]. The authors stated that ERLIC is more suitable for peptide separation in shotgun proteomics. The efficiency of Microsorb 100–10 Amino, Microsorb 300–10 Si, microcrystalline cellulose, and ZIC-HILIC were compared for glycopeptide enrichment in batch mode, while Amide-80 and ZIC-HILIC were compared in a column procedure [22]. The best results of *N*-glycopeptide enrichment were obtained with ZIC-HILIC in the batch mode, whereas ZIC-HILIC and Amide-80 resulted in comparable enrichment of glycopeptides in the column mode [22]. Around three times more glycosylation sites were determined after ERLIC prefractionation of digested mouse brain membrane proteins in comparison to enrichment based on hydrazine chemistry [129]. SPE of glycopeptides on Sepharose CL-6B and click-maltose from a tryptic digest of human immunoglobulin G, ribonuclease B, human a1-acid glycoprotein were compared and superior selectivity of the novel click-maltose material was shown by Yu et al. [220].

6 Conclusions

In the recent years many, efforts have been devoted to the development and improvement of enrichment and separation strategies for glycopeptide analysis. These methods are based on glycopeptide glycan recognition (lectin affinity, hydrazide, and boronic chemistry) or take advantage of the differences in the peptide physicochemical properties due to glycan attachment (HILIC or PGC chromatography). Except for the hydrazide chemistry strategy, these methodologies allow the analysis of the intact as well as the deglycosylated glycopeptides allowing glycopeptide characterization and glycosylation site identification. The present methods can be also classified as biased methods (lectin affinity or TiO_2 HILIC) in which specific glycopeptides are enriched; or as unbiased methods (hydrazide or boronic chemistry, PGC chromatography, or most of HILIC strategies) in which all glycopeptides present in the sample can be captured. Biased strategies are useful not only for glycopeptide enrichment but also for extra sample complexity reduction; however, the glycoproteome coverage is reduced. Thus, the selection of biased or unbiased methods will depend on the approach at hand. Although, theoretically, unbiased methods allow the enrichment/separation of all the glycopeptides present in the samples, they have proven to be complementary. Thus, to achieve a more comprehensive glycoproteome coverage, the combination of different glycopeptide enrichment strategies is recommended. Among the described methodologies, lectin affinity chromatography and hydrazide chemistry are the most commonly employed in glycoproteomic studies. However, in spite of the advantages of these strategies, they are mostly restricted to the analysis of *N*-glycopeptides due to the lack of specific lectins for studying *O*-glycosylation and to the lack of efficient methods to release *O*-glycopeptides from hydrazide supports. To this regard, boronic acid, PGC, or HILIC materials constitute more universal approaches allowing the enrichment and separation of both *N*- and *O*-glycopeptides. However, the applicability of boronic acid and PGC strategies to real samples still needs to be proven. Thus, further progress in glycoproteomic studies is related to the development of more selective and efficient materials for glycopeptides enrichment and separation.

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7 References

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