A Pragmatic Guide to Enrichment Strategies for Mass Spectrometry–Based Glycoproteomics

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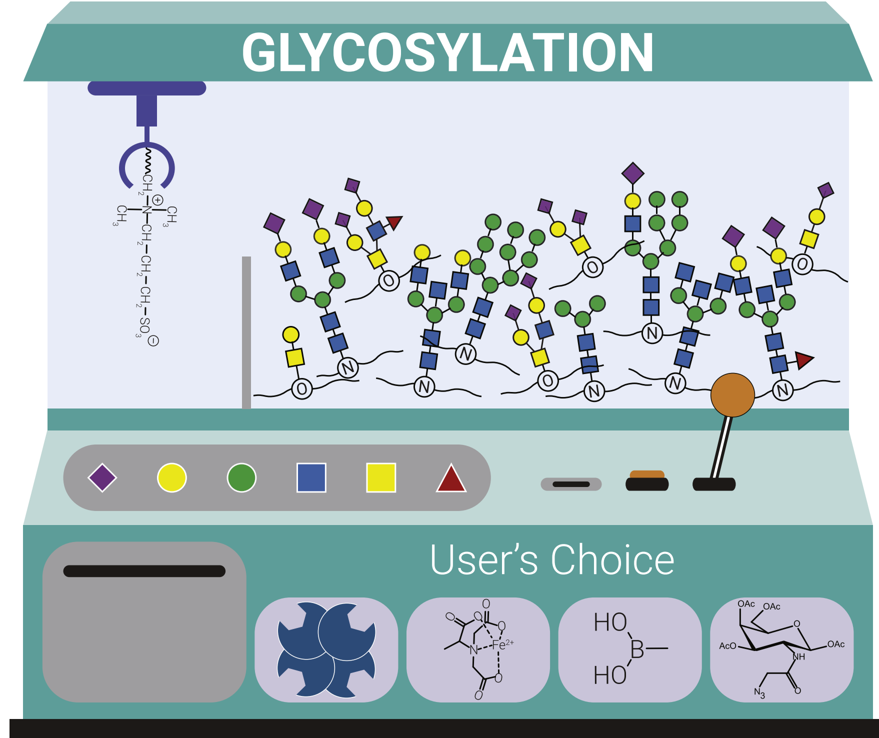
In Brief

Interest in mass spectrometry–based glycoproteomics analysis is increasing because of recent advances in instrumentation and data analysis tools. Such studies can provide a wealth of information across a wide spectrum of glycan classes and biological systems. However, many studies require the choice of an enrichment strategy for glycosylated species prior to analysis to obtain the maximum amount of analytical information. Here, common enrichment strategies are reviewed with strengths and weaknesses, and the practical considerations for various methods are discussed.

Graphical Abstract



Highlights

- Glycosylation is complex and often requires enrichment prior to analysis
- Review of common enrichment strategies for mass spectrometry–based glycoproteomics
- Enrichment methods have practical considerations and experimental implications
- Appropriate enrichment strategies will complement developments in mass spectrometry

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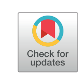

A Pragmatic Guide to Enrichment Strategies for Mass Spectrometry–Based Glycoproteomics

Nicholas M. Riley[1,*], Carolyn R. Bertozzi[1,2], and Sharon J. Pitteri[3,*]

Glycosylation is a prevalent, yet heterogeneous modification with a broad range of implications in molecular biology. This heterogeneity precludes enrichment strategies that can be universally beneficial for all glycan classes. Thus, choice of enrichment strategy has profound implications on experimental outcomes. Here we review common enrichment strategies used in modern mass spectrometry–based glycoproteomic experiments, including lectins and other affinity chromatographies, hydrophilic interaction chromatography and its derivatives, porous graphitic carbon, reversible and irreversible chemical coupling strategies, and chemical biology tools that often leverage bioorthogonal handles. Interest in glycoproteomics continues to surge as mass spectrometry instrumentation and software improve, so this review aims to help equip researchers with the necessary information to choose appropriate enrichment strategies that best complement these efforts.

Mass spectrometry (MS)-based methods are the premier tool for characterizing protein glycosylation, a universal co- and posttranslational modification that exists in all known domains of life (1). This super class of modifications is heterogeneous with several levels of classifications, each of which requires specific analytical considerations (2–6). Glycosylation is primarily defined by the nature of the covalent linkage of mono- or oligosaccharides (*i.e.*, glycans) to polypeptide backbones, usually through nitrogen or oxygen atoms on amino acid side chains (N- and O-glycans, respectively). Hundreds of monosaccharides exist, but only a subset of these are found in commonly observed glycans (7), as shown in Figure 1. Unlike the protein substrates they modify, glycans are not encoded directly in the genome. Instead, their structures are governed by numerous competing and sequentially acting glycosyltransferases and glycosidases that give rise to a diverse pool of glycans constructed from a large number of combinatorial possibilities (8, 9). Glycan expression is also dynamically regulated in response to environmental cues, making precise prediction of glycan structures difficult even with full knowledge of relevant gene products (10–12).

N-glycans and O-glycans also differ significantly in their core structures and the processing steps that define them (13). N-glycans are most often linked through *N*-acetylglucosamine (GlcNAc) at a consensus peptide motif: N-X-S/T, where X is not proline (14–16). Mammalian N-glycans also share a common pentasaccharide core that can then be differentiated into high-mannose, complex, and hybrid types that vary in the connectivity (branching) and composition of their monosaccharide substituents (Fig. 1*B*). Paucimannose N-glycan structures that are subsets of the pentasaccharide core also exist (17). O-Glycosylation, on the other hand, is defined by a number of different monosaccharide linkages, mainly to serine and threonine residues (18–20). Two major classes are intracellular O-GlcNAc, defined by β-GlcNAc with little elongation, and extracellular mucin-type O-GalNAc, defined by an initiating α-GalNAc that can be further decorated into several different core structures (Fig. 1*C*). O-Fucose, O-mannose, O-glucose, and O-xylose classes of O-glycosylation also exist, although most studies currently focus on the O-GlcNAc and O-GalNAc varieties. C- and S-glycosylation through carbon and sulfur atoms on tryptophan and cysteine residues are also known (21–25), but most of the focus here will remain on N- and O-glycosylation in higher mammals. Discussion of glycosylation in other systems, *e.g.*, prokaryotes, plants, and, insects can be found elsewhere (26–30).

Beyond these differences in classes, macroheterogeneity and microheterogeneity are integral facets of glycosylation. Macroheterogeneity refers to occupancy, or the presence or absence of glycans at a glycosite, and this level of regulation is common among all posttranslational modifications (PTMs). Microheterogeneity is a more fascinating, yet more confounding phenomenon of glycosylation where individual glycosites can harbor a range of glycan structural varieties, giving rise to numerous glycoforms that can exist for a single glycosite, much less a single gene product. Differences in glycan composition contribute greatly to glycosite heterogeneity, but structural isomers also contribute, such as conformational isomers (α *versus* β) or linkage differences (*e.g.*, α2,3-linked *versus* α2,6-linked sialic acid). Microheterogeneity can vary

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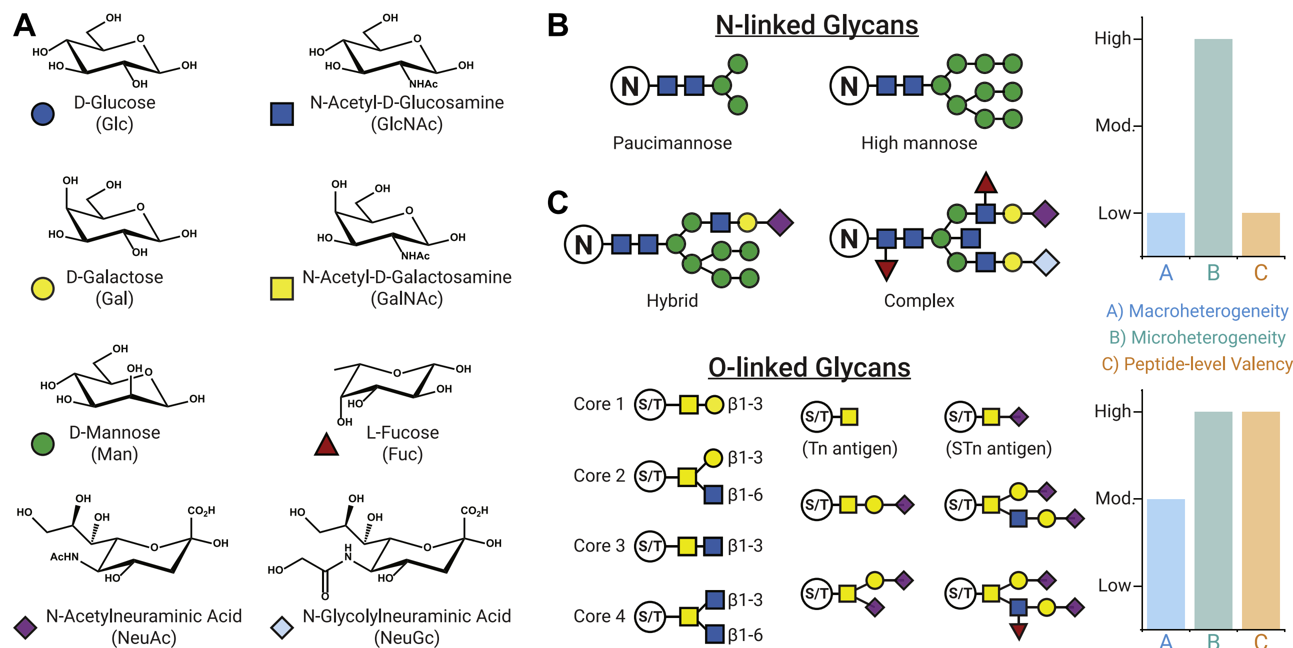

FIG. 1. **Common units in mammalian glycosylation.** *A*, common monosaccharides found in mammalian N- and O-glycans. *B*, N-glycans can be broadly categorized into four classes: paucimannose, high mannose, hybrid, and complex types. *C*, O-glycans can show a high degree of heterogeneity. Here, core 1 to 4 structures of mucin-type O-GalNAc glycans are shown, which can be further elaborated. Common examples of mucin-type O-glycans are provided. Graphs at the right comment generally on the degree of macroheterogeneity, microheterogeneity, and peptide-level valency (*i.e.*, how many glycosites commonly occur in a given glycopeptide) to be expected for both N- and O-glycosites.

glycosite by glycosite or can be somewhat uniform across a given glycoprotein, and differences in glycan structures at a single glycosite can alter biological relevance (31–36). The term "metaheterogeneity" has recently been proposed to describe these levels of variation across glycosites of a given protein (37). Thus, several degrees of characterization exist in glycoproteomic studies, which can range from detection of glycosites following removal of their modifying glycans, to characterization of intact glycopeptides and glycoproteins to interrogate different glycoforms of modified sequences.

Heterogeneity differs for N- and O-glycan classes (Fig. 1, *B–C*). The diversity of N-glycan structures can lead to high microheterogeneity at a given site, but the presence of a glycan at a given glycosite is less dynamic even if the glycan itself differs (meaning macroheterogeneity is relatively low). N-Glycosites often occur with low peptide-level valency, as well, meaning that N-glycopeptides often have only one or two modified N-glycosites. This is biased, of course, by our ability to sequence singly modified N-glycopeptides more readily than multiply-modified species, but even so, many peptides contain only one N-glycan sequon (N-X-S/T) to consider. Mucin-type O-glycosylation also exhibits high site-specific microheterogeneity, in addition to a more pronounced variation in macroheterogeneity (*i.e.*, occupancy) owing to a family of ~20 polypeptide N-acetylgalactosaminyl transferases that differ in expression levels and protein substrate preferences across tissues and physiological conditions (38). Perhaps most challenging to MS-based analysis, mucin-type O-glycosites generally present with a high degree of peptide-level valency in combination with high microheterogeneity, meaning that several O-glycosites occur in close proximity (often on the same glycopeptide) but with potentially different glycans at each site. O-GlcNAc differs substantially from O-GalNAc in its microheterogeneity (because O-GlcNAc modifications are generally the single monosaccharide), but occupancy and peptide-level valency considerations remain similar to O-GalNAc classes.

MS-based approaches can capture these degrees of heterogeneity among all classes of glycosylation, but the presence of glycoforms and the chemistry of glycoproteins relative to nonmodified proteins necessitate enrichment, *i.e.*, the separation of glycoconjugates from nonglycosylated background species, prior to MS analysis. Glycoforms split the signal for molecules with a single glycosite into many different channels, a problem that is further exacerbated for molecules with multiple glycosites. Nonglycosylated sequences do not suffer equivalently from this signal dilution issue, reducing the abundance of individual glycoforms relative to nonmodified species. In other words, a glycosite modified with five different glycans will generate glycopeptides each with one-fifth the abundance of total signal occupied by that glycosite, assuming each glycan modifies the site with equal abundance. Nonmodified peptides from that same protein then appear 5-fold more abundant than each glycopeptide, despite

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their identical biological source. Thus, separation of glycosylated species from nonmodified species greatly improves sensitivity. Furthermore, hydrophobic molecules tend to ionize more efficiently in electrospray ionization, leading to signal suppression of glycosylated sequences that harbor hydrophilic glycan moieties (39–41). Glycan chemistry can further hamper ionization efficiency with the presence of acidic or negatively charged glycans because the majority of MS-based (glyco)proteomics relies on cation analysis in the positive mode. As such, enrichment of glycosylated peptides and proteins has become commonplace in glycoproteomic experiments, even when analyzing relatively simple mixtures.

A number of established enrichment approaches leverage specific properties imparted by glycan moieties. Growing interest in glycobiology, coupled with recent improvements in MS-based instrumentation and software, has also rapidly expanded the collection of enrichment techniques used in glycoproteomic experiments. Here we review principles of common enrichment strategies and discuss their use in glycoproteomic experiments, with an attention paid mainly on efforts to characterize glycopeptides (rather than glycoproteins). We focus on research from the past 5 years in particular, as well. Figure 2 summarizes strategies discussed herein with the mode of enrichment depicted, and Figure 3 provides important considerations that govern what enrichment methods may be best suited for a given application. It is important to emphasize early and often that a universal enrichment strategy does not exist for glycoproteomics. Instead, different approaches can be tailored to glycan classes of interest, meaning researchers must be pragmatic when designing experiments. Our goal here is to provide perspective on which methods are best suited for different needs. We also briefly discuss how these enrichment strategies fit into broader efforts to improve glycoproteome analysis, from sample preparation through data analysis. We direct readers to prior reviews on glycoproteomic enrichment strategies for more historical perspectives (42–50), and we recommend several reviews that focus on N- or O-glycosylation classes more specifically (51–59). We also recognize recent reviews that more categorically address glycopeptide fragmentation methods (60–62), quantitative strategies for glycoproteomics (63, 64), and informatics tools (65–69), all of which make glycoproteomics an exciting and burgeoning field (70–73).

ENDO- AND EXOGLYCOSIDASES

Complex pathways of glycosyltransferases and glycosidases govern the compositions and structures of glycans. Glycosidases, the enzymes that hydrolyze sugar chains, have proven particularly useful as analytical reagents to aid in glycoproteome characterization (74, 75). Although many glycosidases exist, they can be broadly grouped into endoglycosidases, which release glycans from glycoproteins, and exoglycosidases, which release monosaccharides from the nonreducing termini of glycans (*i.e.*, they trim glycan structures but leave them still attached to proteins). The actions and specificities of glycosidases warrant reviews of their own (75–79), but understanding their utility in glycoproteomics is crucial (80). The most widely used glycosidase is PNGaseF, an endoglycosidase that cleaves between the innermost GlcNAc moiety of N-glycans and the asparagine residue to which they are attached (81). High-mannose, complex, and hybrid N-glycans are released by PNGaseF, with some inhibition when glycans contain a core α1,3 fucose (a linkage not found in vertebrates and primarily an insect and plant modification). The PNGaseF cleavage event leaves a deamidated asparagine "scar" at the former N-glycosite that can be detected with high-resolution MS and/or the use of heavy water during the deglycosylation reaction. PNGaseF is generally regarded as the "universal endo-N-glycosidase," even with its few caveats (82), but other endo-N-glycosidases are used in glycoproteomics as well (*e.g.*, EndoH and PNGaseA) (81).

Much of the first wave of MS-based glycoproteomics relied on the release of N-glycans with PNGaseF prior to MS analysis, so-called deglycoproteomics glycoproteomics (51). Here, glycopeptides remain intact through the enrichment stage, but glycans are then removed to make the analytes "deglycopeptides" rather than intact glycopeptides, simplifying LC-MS/MS data acquisition and subsequent informatics requirements. Deglycoproteomic experiments laid the foundation upon which modern glycoproteomics is built, helping to define the scope and relevance of the glycoproteome (83–86), and they still serve as a cornerstone of glycoproteomics technology. These methods are largely limited to N-glycoproteome characterization, however, and remove valuable information about glycosite heterogeneity. They can also introduce some artifacts in glycosite assignment (87), and nonenzymatic readdition of N-glycans following PNGaseF treatment has even been observed (88). As such, a considerable portion of the current glycoproteomic method development focuses on intact glycopeptide analysis rather than deglycopeptides. We discuss the utility of both from the perspective enrichment strategies throughout.

Furthermore, a universal endo-O-glycosidase has not yet been described, although several enzymes can remove O-glycans from peptides following trimming of O-glycans by other exoglycosidases. Alternatively, deglycosylation *via* chemical beta-elimination approaches allow mapping of formerly modified glycosites (89, 90); however, these methods do not reveal the type of beta-eliminated group (*e.g.*, glycan, phosphate) that was present without further experimentation, and they can introduce artifacts from peptide hydrolysis during the beta-elimination process. As such, O-glycoproteomics is more challenging if for nothing else than the preclusion of a facile deglycoproteomics strategy. Characterization of O-glycosites and O-glycans also faces several more challenges discussed below, especially when N-glycans are also present in a sample. Because (the majority of) N-glycans can

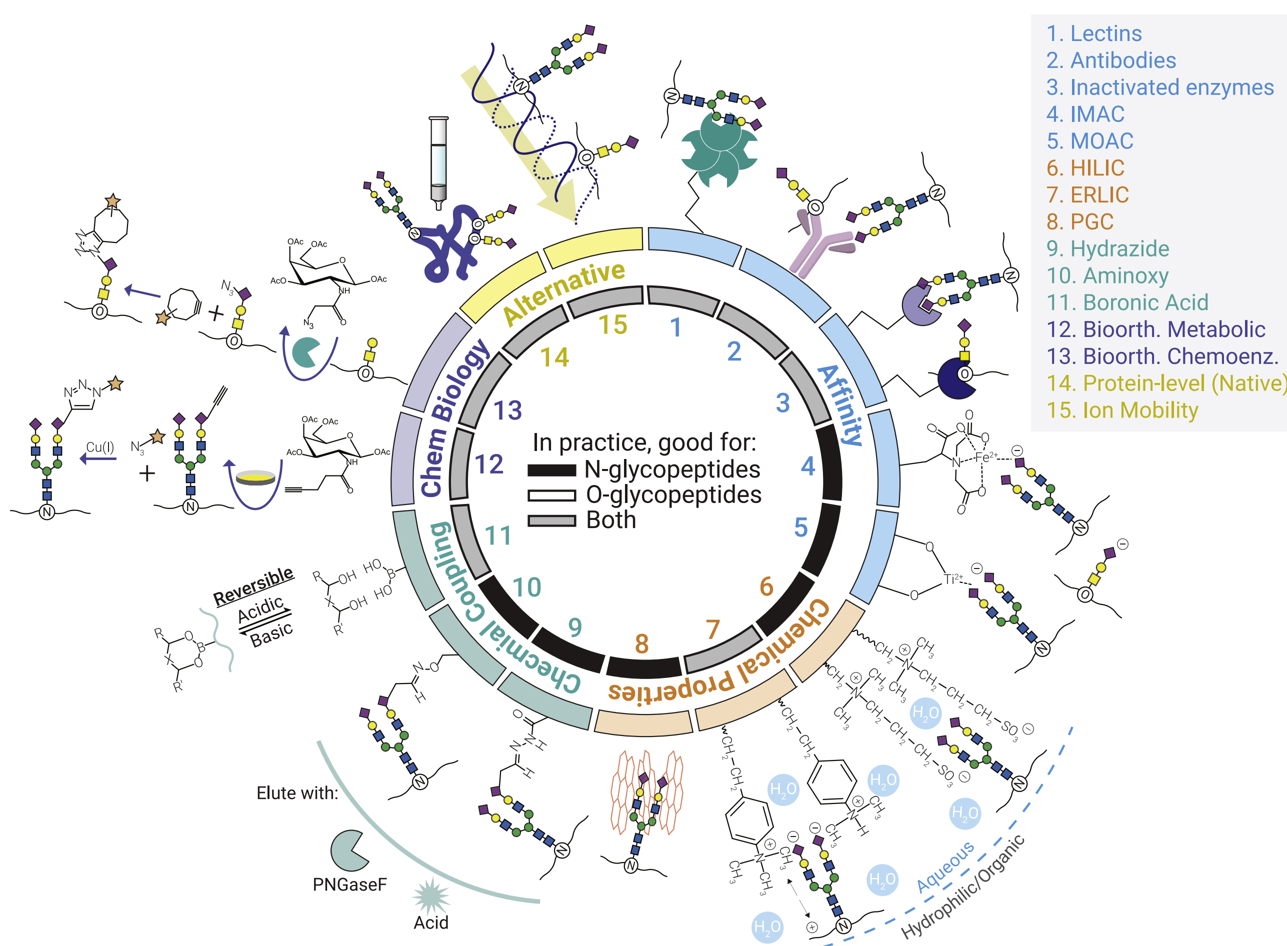

Fig. 2. **A summary of glycosylation enrichment strategies**. Glycosylation is a heterogeneous modification that cannot be fully enriched with a single approach. Glycopeptides can be enriched along multiple axes, including affinity for glycans *via* glycan-binding proteins or charged moieties, chemical properties such as charge or hydrophilicity, chemical coupling of glycans to stationary phases, and incorporation of bio-orthogonal handles through various chemical biology approaches. Alternative approaches, like native mass spectrometry (intact protein-level analysis) and gas-phase separations *via* ion mobility spectrometry are also gaining popularity in glycoproteomics. Here, the inside circle shows the suitability of each approach for N-glycopeptides (*black*), O-glycopeptides (*white*), or both classes (*gray*). The lack of white labels shows that no broad category of method is exclusively suited for O-glycopeptides, although specific implementations may be O-glycosylation oriented (*i.e.*, certain metabolic sugar analogs). Details of the figure include: antibody recognition and inactive glycosidase/glycoprotease recognition of glycan or [glycan + peptide] epitopes, an example of ZIC-HILIC stationary phase, SAX and WAX resin examples for ERLIC (left and right structures, respectively), CuAAC using an alkynyl sugar analog and an azido probe for bioorthogonal metabolic labeling, and SPAAC using an azido sugar analog and a cyclooctyne probe for bioorthogonal chemoenzymatic labeling. CuAAC, copper-catalyzed azide-alkyne cycloadditions; ERLIC, electrostatic repulsion-hydrophilic interaction chromatography; SAX, strong anion exchange; SPAAC, strain-promoted azide-alkyne cycloaddition; WAX, weak anion exchange; ZIC-HILIC, zwitterionic hydrophilic interaction chromatography.

be easily removed with endoglycosidases, most O-glycoproteomics experiments incorporate PNGaseF treatment prior to glycopeptide enrichment and/or elution steps. This not only improves enrichment efficiencies for the often-smaller O-glycans but also facilitates postacquisition data interpretation.

Glycosidases are also essential to glycomics, or the characterization of free oligosaccharides, a complementary and extremely valuable field of analytical glycobiology that is inherently intertwined with glycoproteomics. Several excellent reviews describe fundamental concepts, challenges, and advances in glycomics, which we have space to only tangentially discuss in this work (91–95). Finally, we note that glycosylation heterogeneity described throughout this review, which brings many challenges to glycoproteomics, can be addressed to various degrees by the use of endo- and exoglycosidases to either remove or simplify glycans, respectively. Even so, this reduces the biological context captured in an experiment. Defining glycosylation heterogeneity within a system of interest is often the very point of the glycoproteomic analyses, mandating strategies to characterize intact glycopeptides with biologically relevant glycans attached to their endogenous sites. However, deglycoproteomics may remain the most

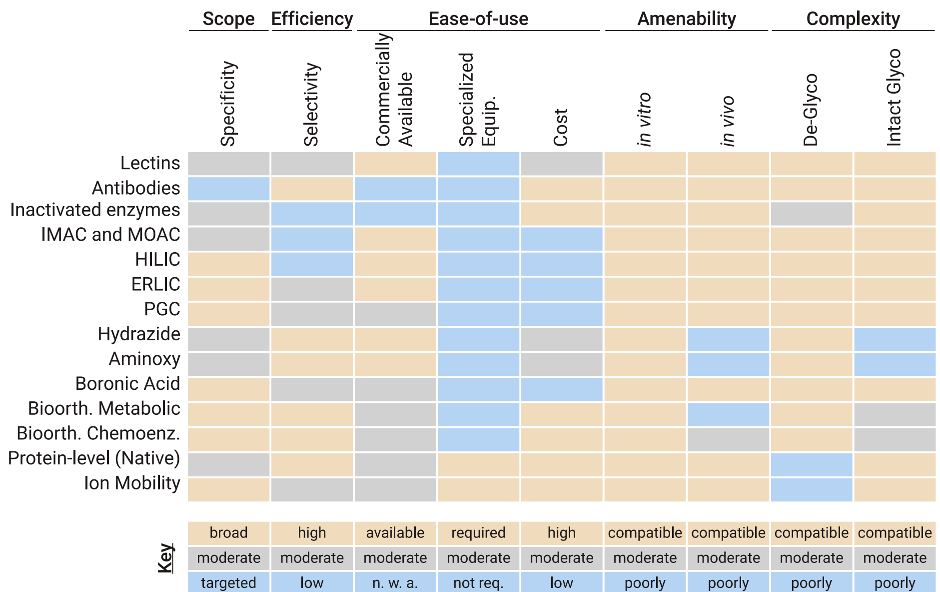

Fig. 3. **Considerations when choosing a glycopeptide enrichment approach**. Success of various glycoproteomic enrichment strategies depends on several experimental conditions. Selection of the appropriate enrichment tool must be evaluated based on criteria that include scope of the experiment (broad *versus* targeted glycan class specificity), enrichment efficiency (*i.e.*, selectivity), the ease of use and implementation, amenability to various biological systems, and the complexity of the analytes to be analyzed (deglycopeptides *versus* intact glycopeptides). The abbreviation "n. w. a." stands for "not widely available," and "poorly" is short for "poorly suited."

advantageous approach when under extremely sample limited conditions, where heterogeneous glycopeptide signal can be condensed into fewer channels *via* deglycopeptides.

LECTINS AND AFFINITY CHROMATOGRAPHIES

Affinity chromatography is the broad term for leveraging specific biochemical interactions of analytes with immobilized ligands to enrich species of interest from background matrices, and it has a long history of use in concert with MS-based proteomics and related approaches (96, 97). In glycoproteomics, lectins have been the affinity chromatography approach of choice for decades because of their recognition of carbohydrates (98–100). These carbohydrate-binding proteins are present in all living organisms and can discriminate between glycan structures with varying degrees of specificity for both glycans and the substrates they modify. Hundreds of lectins, typically bound to support materials such as agarose or POROS (poly(styrene-divinylbenzene)), have been used in glycoproteomic experiments, with a majority of widely used lectins like concanavalin A (ConA), wheat germ agglutinin (WGA), *Sambucus nigra*, ricinus communis agglutinin (RCA), and jacalin coming from plants. Notable examples of mammalian lectins that will not receive due attention here include galectins (which recognize β-galactoside-containing glycans) (101) and sialic acid–binding immunoglobulin-type lectins (Siglecs) (102), which can be used in pulldown assays to define specific ligands.

Lectin specificities can be leveraged to enrich desired glycoforms for glycoproteomic analyses. For example, ConA is known to favor α-mannose-containing glycans such as those found in many N-glycans. Native WGA recognizes GlcNAc and sialic acid residues present in N-glycans and O-GlcNAc glycosylation; however, succinylated WGA improves the specificity for beta-GlcNAc. The most commonly observed specificities across lectins are for galactose, GlcNAc, glucose, mannose, fucose, GalNAc, and Neu5Ac. The use of lectins for enrichment is generally referred to as lectin affinity chromatography (LAC), and often combinations of multiple lectins are used to enrich various classes of glycopeptides from the same sample, *i.e.*, multilectin affinity chromatography (M-LAC) (103).

LAC has been successfully used for a large number of both N- and O-glycoproteomic experiments. WGA is among the more widely used lectins because of its recognition of GlcNAc residues that are found in many different glycan types. Indeed, many efforts that target O-GlcNAc glycosylation use WGA (104–109). In an interesting set of studies, Trinidad, Burlingame, *et al.* (110) used microbore columns packed with WGA conjugated to POROS beads to investigate O-GlcNAc cross talk with phosphorylation in murine synapses. They followed this with work using WGA enrichment to profile both N- and O-glycopeptides from murine synaptosomes, one of the most comprehensive glycosylation profiling efforts to date (111). This experiment, which captured >2500 unique N- and O-linked glycopeptides, demonstrated the broad range of glycan classes WGA can enrich, including both intracellular O-GlcNAc and endoplasmic reticulum and Golgi-derived N- and O-glycopeptides. Of interest, WGA columns have been continued to be used by Medzihradszky and co-workers

to characterize several glycopeptide classes, including sialylated N- and O-glycopeptides (112), mucin-type O-glycopeptides (113), and O-glycopeptides with extended and acetylated sialic acid structures (114, 115).

WGA is often used together with other lectins in M-LAC approaches, where combinations with ConA, jacalin, and RCA lectins are common (116–121). Zielinska *et al.* (83) used ConA, WGA, and RCA lectins for their N-glyco-FASP method that mapped >6300 N-glycosites on >2300 N-glycoproteins from several mouse tissues in deglycoproteomic experiments. Jacalin has also proven useful for O-glycopeptide enrichment, both alone (122) and in combination with WGA (123) or ConA (124). Jacalin generally enriches for core-1 O-glycans with and without sialylation, although it also binds mannose residues. Alternatively, M-LAC can also be accomplished with lectins that are not used as commonly, such as King *et al.* (125) who used *vicia villosa* agglutinin (Tn-antigen) and peanut agglutinin (T-antigen, *i.e.*, the nonextended core-1 O-glycan) to profile native O-glycosites in platelets, plasma, and endothelial cells. Core-1 and core-2 O-glycans are found on glycoproteins found on many different cell types, whereas core-3 and core-4 O-glycans are more restricted to glycoproteins in the gastrointestinal and bronchial tissues (18). As such, lectins targeting core-1 and core-2 O-glycan enrichment are more widely used for general glycoproteome analysis. In another example of lesser-known lectins in M-LAC, Totten *et al.* (126, 127) combined *Aleuria aurantia* lectin (AAL), *Phaseolus vulgaris* leucoagglutinin, and *P. vulgaris* erythroagglutinin to separate core-fucosylated and highly branched glycans. Even still, new lectins with specific binding capacities are being discovered, such as SL2-1 from *Streptomyces rapamycinicus* that binds α1-6 fucosylated N-glycans but not core α1-3 fucosylated N-glycans (128). Thus, the combinatorial space of M-LAC is vast, allowing flexibility for researchers to tailor enrichments to their experimental questions with some degree of specificity.

Even with the examples described above, one challenge with lectins is defining their specificities. Although it is generally accepted that some lectins have broader binding specificities compared with others, establishing these criteria can require meticulous, dedicated characterization of each lectin of interest (129–131). Many structural characteristics combine to define glycan recognition, including networks of hydrogen bonds, van der Waals contacts, ionic bonds, and bridging interactions of water molecules and bivalent cations (132, 133). Furthermore, many lectins exist in oligomeric states that harbor several carbohydrate-binding sites. Avidity through multiple binding events (*i.e.*, multivalency), combined with nonspecific interactions of lectin domains that are not involved with glycan binding (enrichment efficiencies of ~50% glycopeptides relative to nonglycosylated species are common) can complicate the specificities that are empirically observed.

The number of lectins available for glycoproteomic applications can be daunting, which has led to the development of databases to catalog their specificities, structures, and sources. The Glyco3D portal contains three-dimensional structures and information (134), LectinDB provides information mainly about plant lectins (135), and the Lectin Frontier Database documents lectin specificities (136). A recent concerted effort called UniLectin3D has connected many different axes of glyco(bio)informatic data, cross-referencing the aforementioned databases with other repositories, structural biology databanks, and other curated information (132). Resources like these make selection of appropriate LAC conditions a feasible task for most glycoproteomic needs.

As glycoproteomics shifts toward broad characterization efforts that aim to capture large swaths of the glycoproteome, LAC has somewhat fallen out of vogue in favor of other enrichment techniques (see below). In principle, M-LAC approaches could be designed to capture the majority of heterogeneous structures present in a glycoproteome, but the scale needed to accomplish such a task is prohibitive. That said, experiments that seek to characterize glycoproteins and glycoproteomes with defined glycan structures highlight how valuable LAC remains to glycoproteomics. Perhaps there is no better example of this than with the SimpleCell regime described by Clausen and co-workers (137). The genome editing approach to modify glycosylation pathways has enabled tunable glycoproteomes to be expressed on cells in a modular manner (11, 138). With SimpleCell, the COSMC chaperone gene for C1GALT1 is deleted, eliminating elongation of mucin-type O-glycosylation and leaving either a single GalNAc or an STn O-glycan at each glycosite (139–142). This enables enrichment by lectins like *Vicia villosa* agglutinin, which has a strong preference for single GalNAc moieties on serine and threonine residues. Thus, only one or a small subset of lectins are required to enrich the entire genetically truncated O-glycoproteome that has limited O-glycan structures. This idea can be extended to O-mannose glycans, as well, enabling the enrichment of genetically truncated O-mannose glycoproteome with lectins like ConA (143, 144). The benefits of the limited lectin space required for enrichment is also relevant when using exoglycosidases rather than genomic approaches to simplify O-glycans, which can then be enriched with lectins that recognize the truncated structures (145–147).

Beyond naturally occurring lectins, there are also modified glycosidases acting as lectins reported in the literature. One example includes an O-GlcNAcase mutant that binds O-GlcNAc sites with nanomolar affinity (148). Another example includes the engineered α2,3-specific and pan-specific sialidases developed by Lectenz that have affinity and specificity for sialic acid (149, 150). For N-glycoprotein and N-glycopeptide enrichment in particular, Fbs1 carbohydrate-binding protein (which functions in the ubiquitin degradation system) has been engineered with high selectivity for N-glycan pentasaccharide core (Fig. 1, paucimannose structure) in a wide range of N-glycan classes (151). In addition to these

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approaches, nanoparticles with lectin functionalities have also been made to enable glycopeptide enrichment (152, 153), although this has not become widespread. Regardless of the popularity of LAC for MS-based glycoproteomics and related technologies in the coming years, lectins continue to play important roles in a number of other assays used in glycobiology research, as well, including lectin histochemistry, lectin blotting, enzyme-linked lectin assays, and lectin arrays (154).

Antibodies toward glycans and glycosylated proteins can also be used for enrichment (155). O-GlcNAc and O-GalNAc (specifically, the Tn-antigen on MUC1) antibodies have been reported (156–162), although they have limited use in glycoproteomics thus far. Antibodies have also been used for immunoprecipitation of fucosylated N-glycosylation epitopes (163, 164), but the targeted nature of these enrichments limits their broad use for either N- or O-glycoproteomics. Cummings and co-workers have developed smart antiglycan reagents, which are generated by immunizing lampreys (*Petromyzon marinus*) with glycoconjugates to induce secretion of variable lymphocyte receptors as antibodies toward specific glycan epitopes (165, 166). These may prove valuable for MS-based glycoproteomics but so far have mainly been used in microarray formats.

Immobilized Metal Affinity Chromatography and Metal Oxide Affinity Chromatography

Outside of LAC, immobilized metal affinity chromatography (IMAC) and metal oxide affinity chromatography (MOAC) have gained popularity in enriching negatively charged (*i.e.*, sialylated) glycans. Much of the development in IMAC and MOAC methodology grew from efforts to improve phosphoproteomic workflows (167, 168). IMAC is accomplished by chelating transition metal cations (Fe^{3+}, Ga^{3+}, Ti^{4+}, Zr^{4+}, etc.) onto immobilized substrates, whereas MOAC uses transition metals in metal oxide matrices (most commonly titanium dioxide, TiOx). Both techniques leverage the affinity of deprotonated oxygens in phosphate moieties in phosphoryl groups, but this affinity is not exclusive to phosphopeptides. Thus, deprotonated carboxylic acid groups, such as those on aspartic and glutamic acid side chains, are also enriched with IMAC and MOAC. Similarly, these enrichment modalities select for carboxylic acid anions and hydroxyl groups in sialic acids to enable the enrichment of sialylated glycopeptides, in addition to glycopeptides with phosphorylated glycans (*e.g.*, mannose-6-phosphate, M6P).

In 2007, Larsen *et al.* reported enrichment of sialylated glycopeptides from human plasma and saliva using TiOx enrichment with acidic buffer conditions (169–171). They used alkaline phosphatase to avoid copurification of phosphopeptides in this first iteration, but they later utilized TiOx in combination with sequential IMAC elutions with acidic and basic buffers for simultaneous phospho- and glycopeptide enrichment (172). Here, IMAC enrichment is followed by TiOx enrichment to separate multiphosphorylated peptides from monophosphorylated and glycopeptides. These methods have been used to characterize glycoproteomes (and sometimes phosphoproteomes) of stem cell differentiation (173), Alzheimer's-related neuroproteome changes (174), and stimulated rat neurons, where site-specific glycosylation can change after only seconds of depolarization (175). Of importance, others have shown that sialylated N-glycopeptides can be enriched not only by TiOx but also by Fe^{3+}-IMAC (176) and Ti^{4+}-IMAC (177). Coenrichment of phosphopeptides and glycopeptides can also be achieved with hydrophilic interaction chromatography (HILIC, see below) (178, 179) and nanomaterials functionalized with HILIC matrices, metal ions like Ti^{4+} or Ga^{3+}, or both (180–182).

The above-mentioned experiments relied largely on PNGaseF for N-glycan removal following enrichment, enabling characterization of formerly sialylated deglycopeptides. Several groups have extended these IMAC and MOAC enrichment methods for characterization of intact glycopeptides. Glover *et al.* (183) showed that intact N-glycopeptides with both sialic acid- and M6P-containing glycans can be identified from Fe^{3+}-IMAC enrichments, ultimately characterizing ~4000 phosphopeptides and ~1000 N-glycopeptides simultaneously. Subsequently, Huang *et al.* (184) showed that Ti^{4+}-IMAC can also be used for M6P-glycopeptide enrichments. Around the same time, Hu *et al.* (185) showed that thousands of previously unidentified intact sialylated N-glycopeptides could be found in published IMAC-enriched phosphoproteomic data sets. Cho *et al.* (186) found that coenrichment of phosphopeptides and glycopeptides during IMAC enrichment was inevitable no matter what buffer pH conditions were used. They showed that a subsequent enrichment of IMAC elutions with mixed-mode HILIC separations (discussed in detail below) could largely separate phosphopeptide and glycopeptide populations, enabling identification of ~18,000 phosphopeptides and ~3500 intact glycopeptides when using additional offline fractionation prior to MS analysis (186). Exciting recent studies from multiple groups have shown that these combinatorial enrichments can be used to characterized phospho- and glycoproteomes of patient-derived xenograft tumor tissues and extracellular vesicles from human plasma and urine (187–189).

Of interest, differing reports of glycopeptide *versus* phosphopeptide selectivity in IMAC and MOAC methods are still emerging. Although Fe^{3+}-IMAC is known to enrich sialylated glycopeptides as described above, Caval *et al.* (190) reported that a column-based Fe^{3+}-IMAC matrix (opposed to magnetic beads, etc.) was selective for M6P-containing glycans with little coenrichment of sialylated species. They compared this enrichment to Ti^{4+}-IMAC and TiOx enrichments to show this selectivity was specific to their Fe^{3+}-IMAC conditions, indicating there is still much to explore as to why some conditions promote coenrichment of sialylated glycopeptides while others seem more selective for phosphorylated species. As IMAC and MOAC matrices with different metal ions are

developed and applied to phosphoproteome characterization (191), it will be important to characterize which translate best for characterization of various subpopulations of the glycoproteome. In addition, most IMAC and MOAC enrichment studies have focused on N-glycopeptides, so future studies will need to explore its utility for enriching sialylated, sulfated, and otherwise anionic O-glycopeptides, presumably following enzymatic removal of confounding N-glycans. Outside of direct coenrichment of phospho- and glycosylated peptides, IMAC and MOAC enrichments of phosphopeptides also remain relevant enrichment tools for investigating cross talk of phosphorylation and O-GlcNAcylation (192). In all, the IMAC and MOAC methods may not be well suited for sample-limited situations, owing to the coenrichment of phosphopeptides and possible requirements of multiple handling steps. Informatic challenges of searching for cophosphorylated and glycosylated peptides must also be considered. That said, coenrichment could enable low sample loading amounts if specificities between phospho- and glycopeptides can be properly tuned.

HYDROPHILIC INTERACTION CHROMATOGRAPHY

HILIC is an indispensable tool for glycoproteome enrichment and characterization. Glycopeptide enrichments *via* HILIC have been long established and continue to remain popular across the field (193–196). HILIC has utility both as an enrichment mode prior to reversed-phase LC-MS/MS analysis of glycopeptides and as the chromatography mode used in liquid chromatography directly coupled to MS analysis. Our focus here is primarily on HILIC for enrichment prior to data acquisition rather than an online separation during LC-MS/MS, but we direct readers to several excellent resources for HILIC analyses for both glycopeptides and glycoproteins (194–201). As with many of the enrichment approaches in this review, the literature of HILIC applications in glycoproteomics is vast and impossible to cover exhaustively. Instead, we aim to cover the basic principles and highlight demonstrative examples that highlight its utility as well as limitations for glycoproteome characterization. We also note that HILIC enrichment can be coupled with deglycoproteomic analysis, but the majority of recent studies using HILIC and its derivatives have focused on intact glycopeptide characterization.

HILIC enriches glycopeptides *via* the hydrophilic properties imparted by the glycan, although hydrophilic nonglycosylated peptides can also be coenriched (202). HILIC employs semiaqueous mobile phases to create a "water-enriched" layer within a hydrophilic stationary phase. Separation/enrichment is achieved as hydrophilic glycopeptides partition from organic loading buffers into this hydrophilic environment (203, 204). The primary function of the stationary phase is to bind water, and there are many HILIC stationary phases that can be suitable for glycopeptide enrichment (205). The most widely used are zwitterionic HILIC matrices, where electrostatic forces of the charged stationary phase are (partially) counterbalanced by the proximity of opposite charges. This permanent zwitterion reduces secondary electrostatic interactions that can significantly alter HILIC retention on charged stationary phases but enables higher selectivity than neutral HILIC matrices (86).

HILIC mobile phases are an integral part of the stationary phase (*i.e.*, forming the "water layer"), meaning the water fraction of the mobile phase greatly dictates retention. Generally, at least 3% to 5% water is required in any HILIC buffer to retain sufficient hydration of the resin. Elution of glycopeptides is typically achieved with buffers with a water fraction of ~50% to 60%, whereas higher concentrations of organic solvents increase retention of glycopeptides on the column. Several water-miscible organic solvents are compatible with HILIC separations, but the choice of organic solvent has significant effects on glycopeptide enrichment outcomes.

Alagesan *et al.* (206) evaluated the effects of organic mobile phases for HILIC enrichment while also comparing solid-phase extraction (SPE) and Drop-HILIC (*i.e.*, adding HILIC resin to resuspended peptide mixtures) formats. Their results show that many nuances exist in HILIC enrichment, but generally acetonitrile is the most favorable organic solvent to use (*i.e.*, the fewest number of coenriched nonmodified peptides). Isopropyl alcohol and ethanol can be more beneficial for larger, more hydrophobic glycopeptides, but methanol was poorly suited for HILIC enrichment, presumably from disruption of hydrophilic partitioning *via* hydrogen bonding. In addition, they comment on the comprised enrichment efficiency of SPE HILIC in the presence of salts that are common in reduction/alkylation and proteolysis steps to prepare glycopeptides prior to enrichment. Desalting with C18 material is a nearly ubiquitous step in proteomic workflows that can alleviate this problem, but highly hydrophilic glycopeptides can be lost in this step (207). They recommend a chloroform–methanol precipitation to remove salt instead, although precipitation steps can also be prone to some sample loss. In addition, formic acid is widely used in HILIC enrichment protocols to reduce the coenrichment of hydrophilic nonglycosylated peptides, which can introduce artifacts like glycan formylation (208). Thus, even with the success HILIC enrichments provide for glycoproteomics, several factors could yet be optimized.

HILIC enrichments have been used to enrich glycopeptides from a wide range of biological matrices, including human biofluids (*e.g.*, plasma, serum, and milk) (209–214), cancer systems (215–221), other human model systems (222, 223), murine tissues (178, 224–227), pathogens (228–231), and plants (232). HILIC has been used both as stand-alone and in combination with IMAC and MOAC to enrich phospho- and glycopeptides, too, as mentioned above. Even though HILIC is often thought to be suitable for generic glycoproteome interrogations (233), it is generally best suited for glycopeptides

with relatively high glycan-to-peptide ratios by mass, *i.e.*, glycosylated species with large, branching glycans and/or multiply-glycosylated peptides (202). This biases many HILIC applications to N-glycoproteomics, with a disadvantage for paucimannose N-glycans and O-glycopeptides that tend to have smaller glycans (17, 52, 234, 235). Several studies have successfully leveraged HILIC for O-glycopeptide characterization, including human serum and urine (236–239), *Burkholderia* and *Acinetobacter* bacteria (230, 231), and simple mixtures of O-GalNAc peptides (240, 241). Even so, HILIC may not be the most suitable first choice of enrichment method for studies aiming to characterize the O-glycoproteome.

Several groups have described methods to synthesize or fabricate HILIC resins to continue expanding HILIC capabilities. A common property achieved by these materials is hydrophilicity, although the avenues to achieve such properties differ. Materials include cobalt sulfide nanoleaves (242), covalent/metal organic framework approaches (181, 243–246), carbon microspheres (247), and polymeric monoliths functionalized with piperazine (248), among others. As described above, some of these materials can have bifunctional properties to enable HILIC-based glycopeptide enrichment and metal ion–based phosphopeptide enrichment (181, 182, 249).

Electrostatic Repulsion–Hydrophilic Interaction Chromatography

Mixed-mode HILIC approaches have also gained popularity in recent years. In mixed-mode approaches, the hydrophilic stationary phase has an electrostatic charge (rather than the zwitterionic balance), which superimposes electrostatic interactions with analytes on top of hydrophilic partitioning (250). In these so-called electrostatic repulsion–hydrophilic interaction chromatography (ERLIC) methods, a charged ion exchange column is used with HILIC mobile phases (*i.e.*, high concentrations of organic solvents). The goal is to match or complement the polarity of the ion exchange column with the charge of analytes. Under normal aqueous conditions, electrostatic repulsion between the stationary phase and the analytes would exclude analytes from binding/interacting. However, high organic solvents drive hydrophilic interactions between the polar analytes and the water layer of the stationary phase, and analytes can be retained despite the electrostatic repulsion. This balance can then be modulated *via* pH to alter the electrostatic charge of the analytes (251), which can be used to select for specific functional groups. For glycopeptides, ERLIC stationary phases are usually anion exchange (AX) resins (which have a positive charge), and charges of basic amino groups on peptide backbones and acidic sialic acids on glycans are controlled with buffer pH. This extra degree of tunability gives ERLIC some advantages over zwitterionic HILIC, especially for enriching charged (*e.g.*, sialylated, sulfated) glycans that will have electrostatic attraction to AX stationary phases.

AX resins can be made as weak anion exchange, strong anion exchange (SAX), or mixed-mode strong anion exchange (MAX) based on the functional groups used. MAX resins have attenuated anion affinity, *e.g.*, *via* tertiary amines, that can have a broader selectivity for acids than the strong acid preference of SAX resins. The properties of the AX resin can affect glycopeptide retention, so glycoproteomic ERLIC methods generally include a title descriptive of the resin used. Following a study from Cao *et al.* (252) describing SAX-ERLIC for small-scale glycopeptide enrichment, Totten *et al.* (253) described SAX-ERLIC enrichment for N-glycopeptides from human plasma, benchmarking it against M-LAC and HILIC enrichment. The SAX-ERLIC approach generated the most extensive glycopeptide data of the three and yielded over four times as many unique glycopeptides and half as many non-glycosylated peptides as the HILIC method. Even with the selectivity provided, SAX-ERLIC still had an enrichment efficiency of ~50% (ratio of glycopeptide identifications to total identifications). Zacharias *et al.* (254) compared HILIC and ERLIC, too, although they achieved slightly higher identifications with HILIC. They also reported the two methods to be somewhat complementary and performed sequential HILIC-ERLIC enrichments that provide new identifications not found in HILIC-only analyses (254). Cui *et al.* (255) recently expanded SAX-ERLIC investigations with a detailed study into the "sweet spot" of ERLIC mobile phases that would enable the most selective N-glycopeptide enrichment. They reported that 80% acetonitrile outperformed 95% acetonitrile buffers for hydrophilic partitioning in ERLIC methods (this matches previous HILIC reports (206)), improving the specificity for modified *versus* nonmodified peptides. They also saw that trifluoroacetic acid performed better as an ion pairing agent than formic acid for N-glycopeptide enrichment and that elution of N-glycopeptides is mainly governed *via* disruption of hydrophilic interactions through increased water content (rather than coulombic interactions with pH/salts).

Yang *et al.* (256) reported large-scale N-glycoproteomics with MAX-ERLIC enrichments, identifying 10,338 unique N-glycopeptides that correspond to 1163 N-glycosites on 530 glycoproteins. This, and other studies mentioned above, indicates that ERLIC-based approaches are adept for generic N-glycoproteome interrogations, similar to HILIC methods. That said, ERLIC methods likely have a bias toward charged, sialylated glycopeptide enrichment. MAX methods used by the Zhang group (186) were also referenced above, as they enabled simultaneous profiling of both phospho- and glycopeptides. Similarly, Cui *et al.* (255) showed phosphopeptide coenrichment with SAX-ERLIC. Of importance, this indicates that, although ERLIC-based methods may provide broad N-glycoproteome enrichment, N-glycopeptides are not the exclusive species enriched in these methods, and some sensitivity issues may remain owing to lack of selectivity. Also, it is important to note that ERLIC methods can fail to properly enrich some glycan classes, such as fucosylated glycans seen

in a report from Zhou *et al.* (257), showing that combinations of enrichment methods can often be beneficial.

As with the majority of enrichment methods, more data are available for ERLIC methods when characterizing N- rather than O-glycopeptides. ERLIC has the same limitations in glycan-to-peptide mass ratio considerations that HILIC methods do, although this may be overcome to some degree when seeking to enrich negatively charged glycopeptides. Some evidence of ERLIC methods working for O-glycopeptides include a 2017 study from Yang *et al.* (258), which compared HILIC and two different SAX-ERLIC methods for both N- and O-glycopeptides. They reported several important findings, including 1) improved HILIC enrichment of O-glycopeptides following PNGaseF treatment, 2) improved identification of N- and O-glycopeptides with MAX- and SAX-ERLIC, 3) improved O-glycopeptide enrichment with SAX-ERLIC compared with MAX-ERLIC, and 4) compatibility of ERLIC methods with tandem mass tag isobaric labeling. Other studies have also used ERLIC methods for O-glycopeptide enrichment with varying degrees of success (145, 259, 260). Although this is encouraging, more data are likely needed to know how generalizable ERLIC will be for characterizing the O-glycoproteome as methods improve the depth at which the O-glycoproteome can be sampled. Overall, HILIC and its derivatives like ERLIC will continue to be a mainstay in glycopeptide enrichment, and we expect them to be utilized in large-scale quantitative glycoproteomic studies for the foreseeable future.

POROUS GRAPHITIC CARBON

Alternative chromatographic modes that enable retention of polar and hydrophilic species have always been an interest for many bioanalytical fields that traditionally rely on reversed-phase separations. Porous graphitic carbon (PGC), developed to overcome shortcomings of silica-based stationary phases, is a crystalline material composed of sheets of graphitized carbon that are held together through van der Waals interactions in a hexagonal arrangement, although successive layers are not oriented regularly (differentiating it from traditional three-dimensional graphite) (261). PGC retains polar analytes with unique behavior relative to other stationary phases, although the mechanisms of this retention are not fully understood (261, 262). Hydrophobicity and charge contribute to retention, as does molecular shape, which are partially explained through dipole–dipole interactions induced on the surface by charged analytes and electronic repartition on the graphic surface that places an electronic excess at the edges of planes within the PGC structures (261). Whatever the reasons, PGC has proven useful for enriching and separating glycans and glycopeptides (263–270).

PGC can be used both in SPE formats to enrich glycopeptides and in online methods, sometimes in combination with reversed-phase separation, for LC-MS/MS. PGC-SPE formats have yet to be widely adopted for enrichment (271, 272), likely because complicated retention mechanisms make method optimization challenging and less intuitive. That said, PGC has the attractive potential of chromatographically separating isomeric glycopeptides based on their glycan structures, and several studies have utilized online PGC separations to characterize intact glycopeptides (233, 273–280). One challenge is that hydrophobic glycopeptides are difficult to elute from PGC, so most studies using online PGC separations have relied on nonspecific proteases to generate small peptide backbones for glycopeptides with limited hydrophobicity. Coupling of PGC with reversed-phase separations can help (281, 282), but the use of nonspecific proteases still creates an additional layer of peptide backbone heterogeneity on top of the already challenging glycosylation heterogeneity present in the sample. Mechref and colleagues recently reported improved PGC-LC-MS characterization of N-glycopeptides from cleavage- specific protease (trypsin/LysC, GluC, and chymotrypsin) through the use of higher temperatures and basic (rather than acidic) mobile phases (283), although higher temperatures have been reported to improve glycopeptide isomer discrimination for reversed-phase separations, too (284). The potential uses of PGC for glycoproteome characterization are exciting, but it remains to be seen if the technical challenges associated with this unique stationary phase will limit its application to large-scale analyses.

CHEMICAL COUPLING STRATEGIES

Glycans inherently contain functional groups that can be directly targeted for enrichment (285). Historically, hydrazide chemistry has been widely used to profile sialylated glycoconjugates (286). Hydrazine groups (NH_2-NH_2) can selectively react with aldehydes, which do not naturally exist in glycans but can be generated by the periodate oxidation of sialic acids. A wide range of scaffolds can incorporate hydrazide functional groups that can then be leveraged to enrich glycopeptides containing oxidized sialic acids. One of the first applications of hydrazide chemistry for enrichment in glycoproteomics was by Zhang *et al.* (287), who used a solid support matrix functionalized with immobilized hydrazide groups to covalently capture sialylated glycopeptides. PNGaseF was then used to release N-glycopeptides from the solid support, enabling characterization of de-N-glycopeptides.

This approach has been expanded in a number of ways since then, with uses in both N- and O-glycoproteomics (288–297). One recent improvement on the method, called solid phase extraction of *N*-linked glycans and glycosite-containing peptides, tethers glycopeptides to solid support through the peptide N terminus (after blocking other ε-amino groups with guanidination) rather than the glycan (298). Several other steps, including anilination of carboxylic acids, N-glycan release with PNGaseF, deglycopeptide elution *via*

AspN digestion, and separate analysis of enriched intact glycopeptides, ultimately generate information about the total *N*-linked glycan, glycosite-containing peptide, and glycoprotein content of complex samples. Variations of this method has been used for a variety of applications, including characterization of atypical N-glycosites that have N-X-C motifs (299), and it has been recently reported to outperform HILIC and other solid-phase enrichment methods (300). Similar approaches have also been adopted for O-glycopeptide analysis (301–303). These methods are largely enabled by a newly described O-glycoprotease called OpeRATOR, an enzyme from the gut commensal *Akkermansia muciniphila* (304). O-Glycopeptides are released from solid support matrices by OpeRATOR cleavage, which leaves an O-glycosite at the N-terminal peptide residue (259). However, even though the N-terminal residue will be glycosylated, it cannot be assumed to be the only O-glycosite in the sequence, meaning some complications of O-glycopeptide characterization remain (259, 305).

Hydrazide chemistry has also been fundamental to developing strategies to profile glycoproteins exclusively labeled at the cell surface. Analyzing glycoproteomes from whole-cell lysate generates confounding artifacts that complicate determination of what glycoforms are biologically relevant and present on the cell surface. Several approaches have been developed to label cell-surface glycoproteins on live cells, prior to cell lysis and protein extraction (306, 307). One variation functions similarly to the approaches above: sialic acids are oxidized on live cells *via* mild periodate treatment, and aldehyde-containing sialylated glycopeptides are captured on hydrazide beads. However, elution differs in that acidic conditions are used to cleave the linkage of sialic acids to the rest of the glycan, releasing intact (yet asialylated) glycopeptides for analysis (308, 309). The benefits of intact glycopeptide analysis are important, and this opens the method to both N- and O-glycopeptide characterization, but the acidic conditions used for elution can introduce a number of artifacts. A more widely adopted approach called Cell Surface Capture instead labels oxidized sialic acids with biocytin-hydrazide, a bifunctional molecule that can generate biotinylated species (85, 310). Thus, sialylated glycopeptides derived from cell surface glycoproteins can be enriched with streptavidin and eluted *via* PNGaseF release for de-N-glycopeptide analysis. Although this method has thus far been limited to characterization of formerly N-glycosylated peptides, it has been a valuable approach for generating cell surface glycoprotein maps of common mammalian cell lines and is used for a variety of applications (311–322).

Alkoxyamine (aminooxy) compounds also conjugate to aldehydes in a similar manner to hydrazide compounds, making them viable tools for glycoproteomic enrichment. That said, they are far less utilized than hydrazide compounds in glycoproteomics. A notable exception is the PAL method from Paulsen and co-workers, which functions similarly to Cell Surface Capture (323, 324). Thiazolidine chemistry was also recently used for a similar approach (325). Multifunctional probes using hydrazide and aminooxy groups have been used to target ligand–receptor interactions at the cell surface, as well (326–329). These aldehyde-selective chemistries can be expanded further to target other glycan classes if aldehyde groups can be generated on specific monosaccharides. For example, this can be accomplished through enzymatic approaches that use galactose oxidase, which can selectively oxidize galactose and galactose oxidase. Several studies have used galactose oxidase for cell surface profiling *via* hydrazide- and aminooxy-based enrichment approaches (330–335).

Monosaccharides other than sialic acids and galactose can also be targeted with these strategies, although to a limited degree. Rannes *et al.* (336) showed that directed evolution of galactose oxidase could enable oxidation of mannose and GlcNAc residues. Oxidation of the GlcNAc *trans* diol into an aldehyde can enable O-GlcNAc enrichment through hydrazide chemistry (337), although these reactions typically require high concentrations of sodium periodate and high temperatures (337). Even so, hydrazide and aminooxy chemistry methods currently bias strongly toward N-glycoproteome analysis because of the reliance on PNGaseF for elution in a majority of cases, so new strategies will need to emerge to enable similar characterization of the cell-surface O-glycoproteome. Also, sensitivity and selectivity can be influenced by biomolecules that naturally contain carbonyl groups (*e.g.*, metabolites like glycose, pyruvate) and by side reactions that occur during periodate oxidation of glycans (*e.g.*, oxidation of methionine, cysteine, and N-terminal serine and threonine residues) (338).

Reversible Chemical Coupling

Although carbonyl-reactive probes like hydrazide and aminooxy compounds are valuable and popular tools, boronic acid–based enrichments are arguably the more versatile chemical coupling approach for glycopeptide enrichment. Boronic acids can covalently react with *cis*-diols to form five- or six-membered cyclic esters in basic conditions, and, of importance, this reaction is reversible under acidic conditions (339–341). Indeed, glycans contain *cis* 1,2 and 1,3 diols that can conjugate with boronic acid, enabling a selective but reversible covalent glycopeptide enrichment platform (342). Boronic acid–based methods have shown potential as an unbiased large-scale glycoproteomic enrichment strategy (343–348), but glycan–boronic acid interactions are relatively weak. This presents a disadvantage for enriching many low-abundance glycopeptides that may be outcompeted during the enrichment process. Recently, several reports have shown that boronic acid–glycan interactions can be enhanced through screening of boronic acid compounds or through increasing avidity *via* dendrimeric boronic acid–functionalized materials (345, 346, 349–351).

Boronic acid–based enrichments have the potential to characterize both N- and O-glycopeptides. O-Glycopeptide enrichment with boronic acid approaches has been reported

(352), although the interactions with smaller O-glycans may be weaker and thus harder to capture. We anticipate that boronic acid–based enrichments will gain popularity as novel materials continue to improve the strength of boronic acid–glycan interactions and continue to become more available. Furthermore, continued development of reversible covalent modifications, such as a recently described Schiff base hydrolysis strategy (353), will continue to enable intact glycopeptide enrichment. Also of interest are host–guest interactions, which can enable reversible capture of glycosylated species with specificities for monosaccharides, *e.g.*, N-glycolylneuraminic acid (Neu5Gc) and N-acetylneuraminic acid (Neu5Ac) sialic acids (354). Although these have limited application in glycoproteomics thus far, they represent a potentially useful platform for future enrichment modalities.

CHEMICAL BIOLOGY TOOLS

Chemical biology approaches to glycopeptide enrichment seek to add nonendogenous chemical handles to glycans that can be targeted to separate glycosylated species from nonmodified peptides with a high degree of specificity (355, 356). Often these methods use bioorthogonal reactions, *i.e.*, chemical reactions that occur inside of living systems without interfering or interacting with native biochemical processes (357). Chemical handles can be incorporated into glycans metabolically, enzymatically, or through chemical coupling strategies discussed above (*e.g.*, hydrazide chemistry) (358).

Metabolic incorporation of bioorthogonal handles into glycans can be accomplished with chemically functionalized monosaccharides. Azides are typically chosen as the bioorthogonal handle because they are small, unreactive with endogenous cellular components, stable *in vivo*, and easy to add to synthetic sugars (359, 360). Azido-sugars can then be selectively labeled through copper-catalyzed azide-alkyne cycloadditions (CuAAC, *i.e.*, “click” chemistry), strain-promoted azide-alkyne cycloaddition (SPAAC, *i.e.*, “copper-free click” chemistry), or Staudinger ligations (361–366). Alkynes can also be used as the bioorthogonal handle for unnatural monosaccharide incorporation, which are then labeled with azide probes (357, 359, 360).

Glycan biosynthesis pathways can metabolically incorporate unnatural biosynthetic precursors to target specific forms of glycosylation. For example, analogs of ManNAc, *e.g.*, ManNAz (azide handle) and ManNAlk (alkyne handle), get exclusively incorporated as sialic acids, providing an avenue to specifically label sialylated glycoproteins (362, 367, 368). Glycoproteomic experiments have taken advantage of a wide range of azido and alkynyl monosaccharide precursors, including variants of ManNAc, GalNAc, GlcNAc, and fucose (369–378). Although sialylated N- and O-glycans can be specifically labeled, incorporation of other sugars can be more complicated. Fucose variants can label N-glycans (either core fucose or antennary fucose) or O-fucose glycans, but incorporation of fucose derivatives can be limited or even inhibit protein fucosylation (374, 375). GalNAc analogs can be incorporated into N- and mucin-type O-glycans, but GalNAc precursors can also be epimerized into GlcNAc *via* UDP-galactose 4-epimerase (371). This leads to labeling of both GalNAc and GlcNAc moieties when GalNAc analogs are used, complicating labeling selectivity unless UDP-galactose 4-epimerase knockouts or other genetic strategies are pursued. This metabolic cross talk also affects labeling with GlcNAc variants (379, 380), which are generally used for the goal of targeted O-GlcNAc labeling but result in colabeling of GalNAc-containing sugars. That said, O-GlcNAc analogs specific to O-GlcNAc labeling have been described (381–383), enabling profiling of O-GlcNAcylated proteins without confounding GalNAc enrichment. Metabolic labeling strategies have enabled several recent glycoproteomic studies, including N-glycoproteome profiling (384, 385), O-glycoproteome profiling (386–388), N- and O-glycoproteome profiling from the same samples (389–391), cell surface glycoproteomics (392–394), O-GlcNAc-centric glycoproteomics (395–397), and even glycoproteomics in *ex vivo* cultured human tissues (398).

Chemoenzymatic labeling of glycans with bioorthogonal handles, where enzymes append functionalized monosaccharides to native glycans, is a complement to metabolic incorporation (399). Chemoenzymatic labeling of glycans for glycoproteomic enrichment has long been used to target O-GlcNAc, which generally relies on the action of β-1,4-galactosyltransferase 1 (GalT1) in transferring a galactose to terminal GlcNAc residues. GalT1 has been engineered to accept unnatural sugar analogs that can be labeled with aminooxy or click chemistry probes to enable enrichment (400–411). Alternatively, unmodified galactose can be incorporated with wildtype GalT1, galactose oxidase can be used to generate an aldehyde on the appended galactose, and hydrazide chemistry can then be used to enrich chemoenzymatically modified O-GlcNAc residues (412). Sialic acids have also been targeted with *trans*-sialidase chemoenzymatic labeling strategies (413–416), and recently GalNAc (*i.e.*, Tn-antigen) was profiled by adding stable isotope-labeled galactose to terminal GalNAc residues *via* glycosyltransferase C1GalT1 (417). Selective exoenzymatic labeling has also been used to label glycans with biotinylated or azido-labeled nucleotide-sugar analogs *via* ST6Gal1 and ST3Gal1 sialyltransferases, with specificity demonstrated for N- and O-glycans, respectively (418, 419). Some glycotransferases can also be used to selectively add modified monosaccharides to specific epitopes, such as the bacterial acetylgalactosaminyltransferase used by Zhu *et al.* (420) to selectively label the Neu5Ac-α2,3-Gal epitope with GalNAz for sialic acid linkage discrimination. In practice, many of the probes used to enrich labeled glycans following metabolic incorporation can also be used for enrichment for chemoenzymatically labeled glycans. Metabolic incorporation and chemoenzymatic labeling can also be used in concert to profile the cell surface glycoproteome (56).

The biggest advantage of these chemical biology tools is their selectivity, especially because enrichments *via* bioorthogonal reactions can occur under stringent conditions that minimize nonspecific interactions. They can generally be used to profile both N- and O-glycosylation, as well, depending on experimental design. Of importance, metabolic incorporation approaches are compatible with dose- and time-dependent labeling. However, these methods require judicious selection of biological systems (*e.g.*, with controlled epimerase activity) and are limited in their applicability to human tissue and biofluid samples. Furthermore, metabolic incorporation efficiency is often low, likely owing to tolerance limits of varying biosynthetic enzymes for unnatural substrates, which limits the quantitative capacity of metabolic labeling approaches. Conversely, although chemoenzymatic methods provide a high-efficiency approach to glycan labeling, they are generally only suitable for steady-state glycosylation investigations rather than dynamic regulation. Also, developing enzymes that can tolerate unnatural monosaccharide substrates can be difficult. Despite these challenges, chemical glycobiology and chemical biology for glycoproteomics are rapidly developing fields that have generated an exciting number of improvements and novel approaches, even in the past 5 years (421). Many shortcomings have the potential to be addressed and improved upon as efforts continue.

OTHER STRATEGIES

The above approaches comprise the majority of efforts in glycoproteomic enrichment methodology, but other strategies can be equally as valuable. Acetone precipitation, for example, can be tailored to selectively enrich N- and O-glycopeptides (422). More canonical protein purification strategies can also be used to isolate specific glycoproteins of interest for characterization of glycan heterogeneity. When targeting specific proteins, both top-down (endogenous, nonproteolytically digested protein) and bottom-up (proteolytically derived glycopeptides) proteomic strategies can be used in tandem to assign prevalence of various glycoforms. Native MS approaches have proven adept in these scenarios, where glycoform heterogeneity can be observed in high m/z charge state distributions of native-sprayed glycoproteins while specific glycosite assignments are provided by complementary bottom-up analysis (423–432).

Gas-phase enrichment and separation also continue to gain traction in MS-based proteomics as a whole and have recently demonstrated value for glycoproteome characterization (433). Ion mobility spectrometry (IMS) separates ions based on their mobility in a carrier buffer gas and is generally coupled with MS detection to provide collisional cross sections of ions along with their m/z. IMS can be particularly useful for glycopeptides because various glycan structures alter conformations of the proteins and peptides they modify, thereby changing their mobility in IMS and enabling gas-phase separation (434). There are many varieties of IMS that have been used in glycoproteomics (435), and IMS has been used to separate structural isomers of N-glycopeptides (436–440), isomeric O-GlcNAc and O-GalNAc glycopeptides (441–445), O-glycoforms of glycoproteins (446), and glycoforms with different sialic linkages (447, 448).

Of interest, Scott and co-workers recently showed that field asymmetric waveform IMS gas-phase fractionation can enrich short aliphatic glycopeptides from complex mixtures, which are often unseen in HILIC enrichments (234). The field asymmetric waveform IMS source they used enables compensation voltage ramping within the same experiment (449), which provides flexibility to improve access to both N- and O-glycopeptides. Although the method is not IMS based, Alagesan and Kolarich also recently showed that glycopeptide enrichment may not be necessary prior to LC-MS/MS when using dopant solvents in electrospray ionization sweep gases (which are used to support liquid desolvation and focus the Taylor cone) (41). Acetonitrile and acetone increased the signal intensities of synthetic and proteolytically derived IgG glycopeptides by 5- and 2-fold, respectively. These studies are new and require further investigation, but they represent intriguing opportunities to continue to improve sensitivity in glycoproteomic experiments both with and without glycopeptide enrichment prior to MS analysis.

RELATED DEVELOPMENTS IN GLYCOPROTEOMICS

Glycopeptide enrichment is a crucial component to any glycoproteomics workflow, but several considerations in sample preparation, data acquisition, and data analysis also determine experimental outcomes. Perhaps most fundamental is the way proteins are extracted from biological systems for further processing. Proteomic workflows can bias against membrane proteins and highly hydrophilic or hydrophobic subsets of the proteome (450). Several approaches have been developed to address these methodological blind spots, and they should benefit glycoproteomic methods, too. Specifically, sample preparation protocols that permit the use of detergents during protein isolation should improve characterization of membrane-bound glycoproteins, *i.e.*, a substantial portion of the glycoproteome (451–458). Automated sample preparation protocols also promise to streamline glycoproteomics in concert with other “omics,” including glycomics and deglycoproteomics (459). Furthermore, glycosites often occur in regions of proteins that can be undersampled with standard proteomic methodologies that rely on trypsin. Multiple protease approaches have already proven their benefit for glycoproteome characterization (86, 460–464). Even so, these largely rely on canonical proteases like trypsin, chymotrypsin, GluC, and AspN, among others. Some classes of glycosylation, *e.g.*, mucin-type O-glycosylation remain impervious to these proteases. Nonspecific proteases can provide benefits in many cases (238, 281, 465), but data

analysis of glycopeptides from nonspecific proteolysis can prove challenging and sometimes unreliable. Studies describing proteases that specifically target O-glycoproteins are beginning to emerge (301, 302, 466–468), which have the potential to make O-glycoproteomics more tractable despite a current lag behind N-glycoproteome characterization. Of importance, inactive point mutants of these proteases (similar to engineered glycosidases discussed above) can also be valuable tools for selective enrichment of the O-glycoproteome (304, 469).

Even the best-suited enrichment methods lack utility if the glycopeptides they enrich cannot be readily identified with the available MS acquisition and informatics tools. Furthermore, enrichment methods cannot be honed or optimized without proper analysis pipelines to facilitate rapid and informative evaluation of outcomes. Tandem MS methodology is paramount to glycopeptide identification and glycosite characterization, and many efforts have explored appropriate fragmentation methods for N- and O-glycopeptides. Collision-based fragmentation, *e.g.*, beam-type collisional dissociation (sometimes referred to as higher-energy collisional dissociation, HCD), is ubiquitous in MS-based proteomics and is indispensable in glycoproteomics, especially for N-glycopeptides. Stepped collision energy methods have become popular for N-glycopeptides, as they balance both glycan and peptide backbone fragmentation within the same MS/MS spectrum (470–473). Collisional dissociation of O-glycopeptides can still produce glycopeptide identifications, but O-glycosite localization is much more difficult relative to their N-glycosite counterparts. Alternative fragmentation methods, namely, electron-driven dissociation techniques like electron transfer dissociation (ETD) (474) and electron capture dissociation (ECD) (475), are generally needed for O-glycosite characterization because peptide backbone fragments from these methods can retain glycan modifications to pinpoint glycosylated residues even in sequences where many potential glycosites exist. Many modern glycoproteomic methods capitalize on real-time combinations of HCD and ETD scans within the same LC-MS/MS analysis, generally conducted through product-dependent triggering where glycan-specific oxonium ions in "scout" HCD scans trigger subsequent ETD scan(s) for the same precursor ion (476–479). In addition, ETD and ECD methods often benefit from hybrid approaches that use supplemental activation either during or following the electron-driven dissociation event to promote more extensive fragmentation (480, 481). These hybrid methods have been shown to improve both N- and O-glycopeptide characterization (320, 482–488), but they are effectively required for site-specific analyses of O-glycopeptides (52, 113, 114, 305, 473). As these nuances between N- and O-glycopeptides continue to be investigated, new acquisitions schemes, such as data-independent acquisition methods (489–494), are beginning to emerge that have the potential to enable consistent and reproducible characterization of larger and larger subsets of the glycoproteome. Negative-mode approaches that enable analysis of glycopeptide anions are also valuable (495) and will likely contribute to improved characterization of sialylated, sulfated, and otherwise acidic glycopeptides as methods continue to improve (496–499).

Informatics tools to interpret information-rich glycopeptide fragmentation spectra are equally as essential. Glycosite microheterogeneity makes glycopeptide spectral analysis a far more intricate challenge than other PTMs. Search algorithms must be able to consider a larger number of potential glycoforms for each peptide based on glycan composition libraries that function as variable modifications. This search space issue is even more pronounced for O-glycopeptides, which often harbor multiple glycosylated residues among several potential glycosite candidates. A growing list of academic and commercial software is making glycoproteomics more accessible than ever (65–69). Among the most popular are Byonic (commercial) and pGlyco (academic), each with their own strengths (224, 500). Particularly exciting are the recently described open-search approaches that not only are more flexible with respect to the glycan composition databases required for analysis (208, 501–503) but also have the ability to greatly improve spectral processing speeds, a major concern when managing many possible glycoforms considerations. Informatic workflows will also benefit from the incorporation of retention time metrics, which has recently been shown to improve N-glycopeptide assignment (504).

As data acquisition and spectral identification methods mature, quantitative strategies that have benefited MS-based proteomics for nearly 2 decades are beginning to make inroads to enable quantitative glycoproteomics (505–507). Label-free quantitation is the most widely used quantitative strategy in standard proteomics and has shown utility for glycoproteomics. That said, reproducibility of sample preparation and enrichments are paramount for label-free quantitation approaches, a challenge that is still being addressed in glycoproteomic workflows (508). As such, stable isotope labeling strategies, including metabolic labeling and isobaric labeling, may prove particularly useful as quantitative glycoproteomics comes of age (63). Studies to investigate best practices for isobaric label-centric quantitative glycoproteomic data are emerging, including how to effectively choose ions for synchronous precursor selection for minimal coisolation interference in multiplexed experiments (509–512). The quantitative approaches selected for glycoproteomics experiments will continue to forge both data acquisition strategies to best acquire accurate quantitative data and also the informatic pipelines to extract and compare quantitative information.

GLYCOPROTEOMICS WITHOUT ENRICHMENT

Indeed, it is possible to characterize glycopeptides without enriching them first, which would eliminate some of the compromises required when choosing enrichment methods.

ASBMB

Glycopeptides are detectable in standard proteomics runs where no enrichment has been performed, even in complex lysates. One simple test to evaluate the presence of glycopeptides in a sample is to generate an extracted ion chromatogram for 204.0867 m/z, the characteristic HexNAc oxonium ion. The product-dependent triggering methods described above (476–479) can be particularly valuable in these cases to facilitate collection of MS/MS scans with longer ion accumulation times, specific dissociation parameters, and higher-resolution settings, as needed, upon the observation of pertinent oxonium ions. That said, the majority of studies that forgo enrichment characterize relatively simple mixtures of glycopeptides, *e.g.*, glycopeptide standards or glycopeptides derived from purified standard glycoproteins or some form of protein-level enrichment prior to proteolysis (for an example among several, see reference 475). An optimistic outlook suggests that gas-phase enrichment strategies described above, including data-independent acquisition methods, may mitigate some need for preacquisition enrichment in more complex studies, but more work is required to realize such a goal.

CONCLUSIONS

There is currently not a single enrichment method that fully captures the diversity of the glycoproteome. This contrasts with PTMs like phosphorylation or acetylation, where enrichments suffer less from inherent biases. The collection of enrichment strategies described here captures the breadth of approaches needed to interrogate the many facets of protein glycosylation, and even this work is not exhaustive. The size of the analytical toolkit for glycoproteome characterization reflects its heterogeneity and intricacy, which both challenge what biological insights can currently be gleaned in glycoproteomic experiments and also drive innovation. Rather than attempt to capture all that exists in the purview of glycosylation in a single experiment, researchers must continue to judiciously pursue questions about glycobiology that can be honed with specific strategies. As described throughout, combinations of enrichment methods can be creatively synthesized when understanding the strengths and limitations of each approach. The coming years are poised to offer rapid development of glycoproteomic technology. We expect that developments in glycopeptide and glycoprotein enrichment strategies will match the advances of ever-improving instrumentation and software and that exciting new enrichment modalities will continue to drive insight into glycoproteome regulation.

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Abbreviations—The abbreviations used are: AAL, *Aleuria aurantia* lectin; AX, anion exchange; ConA, concanavalin A; CuAAC, *i.e.*, "click" chemistry, copper-catalyzed azide-alkyne cycloadditions; DIA, data-independent acquisition; ECD, electron capture dissociation; ERLIC, electrostatic repulsion-hydrophilic interaction chromatography; ETD, electron transfer dissociation; GalT1, β-1,4-galactosyltransferase 1; HCD, higher-energy collisional dissociation; HILIC, hydrophilic interaction chromatography; IMAC, immobilized metal affinity chromatography; IMS, ion mobility spectrometry; LAC, lectin affinity chromatography; M-LAC, multi-lectin affinity chromatography; M6P, mannose-6-phosphate; ManNAz, *N*-azidoacetylmannosamine; MAX, mixed-mode strong anion exchange; MOAC, metal oxide affinity chromatography; MS, mass spectrometry; Neu5Ac, *N*-acetylneuraminic acid; Neu5Gc, *N*-glycolylneuraminic acid; PGC, porous graphitic carbon; PTMs, post-translational modifications; RCA, ricinus communis agglutinin; SAX, strong anion exchange; Siglecs, sialic acid–binding immunoglobulin-type lectins; SPAAC, *i.e.*, "copper-free click" chemistry, strain-promoted azide-alkyne cycloaddition; SPE, solid-phase extraction; WAX, weak anion exchange; WGA, wheat germ agglutinin; ZIC-HILIC, zwitterionic HILIC.

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REFERENCES

1. Varki, A. (2011) Evolutionary forces shaping the Golgi glycosylation machinery: why cell surface glycans are universal to living cells. *Cold Spring Harb. Perspect. Biol.* **3**, 1–14
2. Spiro, R. G. (2002) Protein glycosylation: nature, distribution, enzymatic formation, and disease implications of glycopeptide bonds. *Glycobiology* **12**, 483–491
3. Ohtsubo, K., and Marth, J. D. (2006) Glycosylation in cellular mechanisms of health and disease. *Cell* **126**, 855–867
4. Zhang, P., Woen, S., Wang, T., Liau, B., Zhao, S., Chen, C., Yang, Y., Song, Z., Wormald, M. R., Yu, C., and Rudd, P. M. (2016) Challenges of glycosylation analysis and control: an integrated approach to producing optimal and consistent therapeutic drugs. *Drug Discov. Today* **21**, 740–765
5. Leymarie, N., and Zaia, J. (2012) Effective use of mass spectrometry for glycan and glycopeptide structural analysis. *Anal. Chem.* **84**, 3040–3048
6. De Leoz, M. L. A., Duewer, D. L., Fung, A., Liu, L., Yau, H. K., Potter, O., Staples, G. O., Furuki, K., Frenkel, R., Hu, Y., Sosic, Z., Zhang, P.,

Altmann, F., Grunwald-Grube, C., Shao, C., *et al*. (2020) NIST interlaboratory study on glycosylation analysis of monoclonal antibodies: comparison of results from diverse analytical methods. *Mol. Cell. Proteomics* **19**, 11–30
7. Tiemeyer, M., Aoki, K., Paulson, J., Cummings, R. D., York, W. S., Karlsson, N. G., Lisacek, F., Packer, N. H., Campbell, M. P., Aoki, N. P., Fujita, A., Matsubara, M., Shinmachi, D., Tsuchiya, S., Yamada, I., *et al*. (2017) GlyTouCan: an accessible glycan structure repository. *Glycobiology* **27**, 915–919
8. Moremen, K. W., Tiemeyer, M., and Nairn, A. V. (2012) Vertebrate protein glycosylation: diversity, synthesis and function. *Nat. Rev. Mol. Cell Biol.* **13**, 448–462
9. Haltiwanger, R. S., and Lowe, J. B. (2004) Role of glycosylation in development. *Annu. Rev. Biochem.* **73**, 491–537
10. Reily, C., Stewart, T. J., Renfrow, M. B., and Novak, J. (2019) Glycosylation in health and disease. *Nat. Rev. Nephrol.* **15**, 346–366
11. Narimatsu, Y., Joshi, H. J., Nason, R., Van Coillie, J., Karlsson, R., Sun, L., Ye, Z., Chen, Y. H., Schjoldager, K. T., Steentoft, C., Furukawa, S., Bensing, B. A., Sullam, P. M., Thompson, A. J., Paulson, J. C., *et al*. (2019) An atlas of human glycosylation pathways enables display of the human glycome by gene engineered cells. *Mol. Cell* **75**, 394–407.e5
12. Freeze, H. H., and Ng, B. G. (2011) Golgi glycosylation and human inherited diseases. *Cold Spring Harb. Perspect. Biol.* **3**, 1–22
13. Varki, A., Cummings, R. D., Esko, J. D., Stanley, P., Hart, G. W., Aebi, M., Darvill, A. G., Kinoshita, T., Packer, N. H., Prestegard, J. H., Schnaar, R. L., and Seeberger, P. H. (2015) *Essentials of Glycobiology*. Cold Spring Harbor Laboratory Press, Long Island, New York
14. Aebi, M. (2013) N-linked protein glycosylation in the ER. *Biochim. Biophys. Acta* **1833**, 2430–2437
15. Pasing, Y., Sickmann, A., and Lewandrowski, U. (2012) N-glycoproteomics: mass spectrometry-based glycosylation site annotation. *Biol. Chem* **393**, 249–258
16. Stanley, P., Taniguchi, N., and Aebi, M. (2017). In: Varki, A., Cummings, R. D., Esko, J. D., Stanley, P., Hart, G. W., Aebi, M., Darvill, A. G, Kinoshita, T., Packer, N. H., Prestegard, J. H., Schnaar, R. L., Seeberger, P. H., eds. *Essentials of Glycobiology*, Cold Spring Harbor Laboratory Press, Long Island, New York
17. Loke, I., Østergaard, O., Heegaard, N. H. H., Packer, N. H., and Thaysen-Andersen, M. (2017) Paucimannose-rich N-glycosylation of spatiotemporally regulated human neutrophil elastase modulates its immune functions. *Mol. Cell. Proteomics* **16**, 1507–1527
18. Brockhausen, I., and Stanley, P. (2017) Chapter 10 O-GalNAc glycans. *Essentials Glycobiology* **1**, 1–9
19. Haltiwanger, R. S., Wells, L., Freeze, H. H., and Stanley, P. (2017) Other classes of eukaryotic glycans. In: *Essentials Glycobiology*, 3rd Ed. 013, Chapter 13, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
20. Zachara, N., Akimoto, Y., and Hart, G. W. (2017) Chapter 19 the O-GlcNAc modification. In: *Essentials Glycobiology*, 3rd Ed, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY: 1–15
21. Hofsteenge, J., Müller, D. R., de Beer, T., Löffler, A., Richter, W. J., and Vliegenthart, J. F. G. (1994) New type of linkage between a carbohydrate and a protein: C-glycosylation of a specific tryptophan residue in human RNase us. *Biochemistry* **33**, 13524–13530
22. Furmanek, A., and Hofsteenge, J. (2000) Protein C-mannosylation: facts and questions. *Acta Biochim. Pol.* **47**, 781–789
23. Venugopal, H., Edwards, P. J. B., Schwalbe, M., Claridge, J. K., Libich, D. S., Stepper, J., Loo, T., Patchett, M. L., Norris, G. E., and Pascal, S. M. (2011) Structural, dynamic, and chemical characterization of a novel S-glycosylated bacteriocin. *Biochemistry* **50**, 2748–2755
24. Stepper, J., Shastri, S., Loo, T. S., Preston, J. C., Novak, P., Man, P., Moore, C. H., Havlíček, V., Patchett, M. L., and Norris, G. E. (2011) Cysteine S-glycosylation, a new post-translational modification found in glycopeptide bacteriocins. *FEBS Lett.* **585**, 645–650
25. Maynard, J. C., Burlingame, A. L., and Medzihradszky, K. F. (2016) Cysteine S-linked N-acetylglucosamine (S-GlcNAcylation), a new post-translational modification in mammals. *Mol. Cell. Proteomics* **15**, 3405–3411
26. Nothaft, H., and Szymanski, C. M. (2010) Protein glycosylation in bacteria: sweeter than ever. *Nat. Rev. Microbiol.* **8**, 765–778
27. Dell, A., Galadari, A., Sastre, F., and Hitchen, P. (2010) Similarities and differences in the glycosylation mechanisms in prokaryotes and eukaryotes. *Int. J. Microbiol.* **2010**, 148178
28. Eichler, J., and Koomey, M. (2017) Sweet new roles for protein glycosylation in prokaryotes. *Trends Microbiol.* **25**, 662–672
29. Strasser, R. (2016) Plant protein glycosylation. *Glycobiology* **26**, 926–939
30. Walski, T., De Schutter, K., Van Damme, E. J. M., and Smagghe, G. (2017) Diversity and functions of protein glycosylation in insects. *Insect Biochem. Mol. Biol.* **83**, 21–34
31. Wu, D., Struwe, W. B., Harvey, D. J., Ferguson, M. A. J., and Robinson, C. V. (2018) N-glycan microheterogeneity regulates interactions of plasma proteins. *Proc. Natl. Acad. Sci. U. S. A.* **115**, 8763–8768
32. Martin, T. C., Šimurina, M., Zabczynska, M., Kavur, M., Rydlewska, M., Pezer, M., Kozłowska, K., Burri, A., Vilaj, M., Turek-Jabrocka, R., Krnjajić-Tadijanović, M., Trofimiuk-Müldner, M., Ugrina, I., Lityn, A., Hubalewska-Dydejczyk, A., *et al*. (2020) Decreased immunoglobulin G core fucosylation, a player in antibody-dependent cell-mediated cytotoxicity, is associated with autoimmune thyroid diseases. *Mol. Cell. Proteomics* **19**, 774–792
33. Brücher, D., Franc, V., Smith, S. N., Heck, A. J. R., and Plückthun, A. (2020) Malignant tissues produce divergent antibody glycosylation of relevance for cancer gene therapy effectiveness. *MAbs* **12**, 1792084
34. Parsons, T. B., Struwe, W. B., Gault, J., Yamamoto, K., Taylor, T. A., Raj, R., Wals, K., Mohammed, S., Robinson, C. V., Benesch, J. L. P., and Davis, B. G. (2016) Optimal synthetic glycosylation of a therapeutic antibody. *Angew. Chem. Int. Ed. Engl.* **55**, 2361–2367
35. Struwe, W. B., and Robinson, C. V. (2019) Relating glycoprotein structural heterogeneity to function – insights from native mass spectrometry. *Curr. Opin. Struct. Biol.* **58**, 241–248
36. Khatri, K., Klein, J. A., White, M. R., Grant, O. C., Leymarie, N., Woods, R. J., Hartshorn, K. L., and Zaia, J. (2016) Integrated omics and computational glycobiology reveal structural basis for influenza a virus glycan microheterogeneity and host interactions. *Mol. Cell. Proteomics* **15**, 1895–1912
37. Čaval, T., Heck, A. J. R., and Reiding, K. R. (2021) Meta-heterogeneity: evaluating and describing the diversity in glycosylation between sites on the same glycoprotein. *Mol. Cell. Proteomics* **20**, 100010
38. Bennett, E. P., Mandel, U., Clausen, H., Gerken, T. A., Fritz, T. A., and Tabak, L. A. (2012) Control of mucin-type O-glycosylation: a classification of the polypeptide GalNAc-transferase gene family. *Glycobiology* **22**, 736–756
39. Wilm, M. (2011) Principles of electrospray ionization. *Mol. Cell. Proteomics* **10**, M111.009407
40. Stavenhagen, K., Hinneburg, H., Thaysen-Andersen, M., Hartmann, L., Silva, D. V., Fuchser, J., Kaspar, S., Rapp, E., Seeberger, P. H., and Kolarich, D. (2013) Quantitative mapping of glycoprotein micro-heterogeneity and macro-heterogeneity: an evaluation of mass spectrometry signal strengths using synthetic peptides and glycopeptides. *J. Mass Spectrom.* **48**, 627–639
41. Alagesan, K., and Kolarich, D. (2019) To enrich or not to enrich: enhancing (glyco)peptide ionization using the CaptiveSpray nanoBooster^{TM}. *bioRxiv*. https://doi.org/10.1101/597922
42. Thaysen-Andersen, M., Larsen, M. R., Packer, N. H., and Palmisano, G. (2013) Structural analysis of glycoprotein sialylation-part I: pre-LC-MS analytical strategies. *RSC Adv.* **3**, 22683–22705
43. Thaysen-Andersen, M., and Packer, N. H. (2014) Advances in LC-MS/MS-based glycoproteomics: getting closer to system-wide site-specific mapping of the N- and O-glycoproteome. *Biochim. Biophys. Acta* **1844**, 1437–1452
44. Zhu, R., Song, E., Hussein, A., Kobeissy, F. H., and Mechref, Y. (2017). In: *Methods in Molecular Biology*, Humana Press Inc, Clifton, NJ: 213–227
45. Gaunitz, S., Nagy, G., Pohl, N. L. B., and Novotny, M. V. (2017) Recent advances in the analysis of complex glycoproteins. *Anal. Chem.* **89**, 389–413
46. Mechref, Y., and Muddiman, D. C. (2017) Recent advances in glycomics, glycoproteomics and allied topics. *Anal. Bioanal. Chem.* **409**, 355–357
47. Chen, C. C., Su, W. C., Huang, B. Y., Chen, Y. J., Tai, H. C., and Obena, R. P. (2014) Interaction modes and approaches to glycopeptide and glycoprotein enrichment. *Analyst* **139**, 688–704

48. Wohlgemuth, J., Karas, M., Eichhorn, T., Hendriks, R., and Andrecht, S. (2009) Quantitative site-specific analysis of protein glycosylation by LC-MS using different glycopeptide-enrichment strategies. *Anal. Biochem.* **395**, 178–188
49. Zhu, R., Zacharias, L., Wooding, K. M., Peng, W., and Mechref, Y. (2017). In: *Methods in Enzymology*, Academic Press Inc, Cambridge, MA: 397–429
50. Nilsson, J., Halim, A., Grahn, A., and Larson, G. (2013) Targeting the glycoproteome. *Glycoconj. J.* **30**, 119–136
51. Thaysen-Andersen, M., Packer, N. H., and Schulz, B. L. (2016) Maturing glycoproteomics technologies provide unique structural insights into the N-glycoproteome and its regulation in health and disease. *Mol. Cell. Proteomics* **15**, 1773–1790
52. Darula, Z., and Medzihradszky, K. F. (2018) Analysis of mammalian O-glycopeptides - we have made a good start, but there is a long way to go. *Mol. Cell. Proteomics* **17**, 2–17
53. Khoo, K. H. (2019) Advances toward mapping the full extent of protein site-specific O-GalNAc glycosylation that better reflects underlying glycomic complexity. *Curr. Opin. Struct. Biol.* **56**, 146–154
54. Levery, S. B., Steentoft, C., Halim, A., Narimatsu, Y., Clausen, H., and Vakhrushev, S. Y. (2015) Advances in mass spectrometry driven O-glycoproteomics. *Biochim. Biophys. Acta* **1850**, 33–42
55. Yu, A., Zhao, J., Peng, W., Banazadeh, A., Williamson, S. D., Goli, M., Huang, Y., and Mechref, Y. (2018) Advances in mass spectrometry-based glycoproteomics. *Electrophoresis* **39**, 3104–3122
56. Xiao, H., Sun, F., Suttapitugsakul, S., and Wu, R. (2019) Global and site-specific analysis of protein glycosylation in complex biological systems with mass spectrometry. *Mass Spectrom. Rev*. **38**, 356–379
57. Mulagapati, S., Koppolu, V., and Raju, T. S. (2017) Decoding of O-linked glycosylation by mass spectrometry. *Biochemistry* **56**, 1218–1226
58. You, X., Qin, H., and Ye, M. (2018) Recent advances in methods for the analysis of protein o-glycosylation at proteome level. *J. Sep. Sci.* **41**, 248–261
59. Dang, L., Jia, L., Zhi, Y., Li, P., Zhao, T., Zhu, B., Lan, R., Hu, Y., Zhang, H., and Sun, S. (2019) Mapping human N-linked glycoproteins and glycosylation sites using mass spectrometry. *Trends Analyt. Chem.* **114**, 143–150
60. Yang, Y., Franc, V., and Heck, A. J. R. (2017) Glycoproteomics: a balance between high-throughput and in-depth analysis. *Trends Biotechnol.* **35**, 598–609
61. Reiding, K. R., Bondt, A., Franc, V., and Heck, A. J. R. (2018) The benefits of hybrid fragmentation methods for glycoproteomics. *Trends Analyt. Chem.* **108**, 260–268
62. Nilsson, J. (2016) Liquid chromatography-tandem mass spectrometry-based fragmentation analysis of glycopeptides. *Glycoconj. J.* **33**, 261–272
63. Delafield, D. G., and Li, L. (2021) Recent advances in analytical approaches for glycan and glycopeptide quantitation. *Mol. Cell. Proteomics* **20**, 100054
64. Ahn, Y. H., Kim, J. Y., and Yoo, J. S. (2015) Quantitative mass spectrometric analysis of glycoproteins combined with enrichment methods. *Mass Spectrom. Rev.* **34**, 148–165
65. Abrahams, J. L., Taherzadeh, G., Jarvas, G., Guttman, A., Zhou, Y., and Campbell, M. P. (2020) Recent advances in glycoinformatic platforms for glycomics and glycoproteomics. *Curr. Opin. Struct. Biol.* **62**, 56–69
66. Hu, H., Khatri, K., Klein, J., Leymarie, N., and Zaia, J. (2016) A review of methods for interpretation of glycopeptide tandem mass spectral data. *Glycoconj. J.* **33**, 285–296
67. Hu, H., Khatri, K., and Zaia, J. (2017) Algorithms and design strategies towards automated glycoproteomics analysis. *Mass Spectrom. Rev.* **36**, 475–498
68. Cao, W., Liu, M., Kong, S., Wu, M., Zhang, Y., and Yang, P. (2021) Recent advances in software tools for more generic and precise intact glycopeptide analysis. *Mol. Cell. Proteomics* **20**, 100060
69. Tsai, P.-L., and Chen, S.-F. (2017) A brief review of bioinformatics tools for glycosylation analysis by mass spectrometry. *Mass Spectrom.* **6**, S0064
70. Doerr, A. (2012) Glycoproteomics. *Nat. Methods* **9**, 36
71. Sheeley, D. M., Azadi, P., and Pohl, N. L. (2019) Emerging technologies to bring glycoproteomics within reach. *J. Biomol. Tech.* **30**, S48
72. Suttapitugsakul, S., Sun, F., and Wu, R. (2020) Recent advances in glycoproteomic analysis by mass spectrometry. *Anal. Chem.* **92**, 267–291
73. Narimatsu, H., Kaji, H., Vakhrushev, S. Y., Clausen, H., Zhang, H., Noro, E., Togayachi, A., Nagai-Okatani, C., Kuno, A., Zou, X., Cheng, L., Tao, S. C., and Sun, Y. (2018) Current technologies for complex glycoproteomics and their applications to biology/disease-driven glycoproteomics. *J. Proteome Res.* **17**, 4097–4112
74. Rudd, P., Karlsson, N. G., Khoo, K.-H., and Packer, N. H. (2017) Glycomics and glycoproteomics. In: *Essentials Glycobiology*, 3rd Ed. Chapter 51, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
75. Kobata, A. (2013) Exo- and endoglycosidases revisited. *Proc. Jpn. Acad. Ser. B Phys. Biol. Sci.* **89**, 97–117
76. Crout, D. H. G., and Vic, G. (1998) Glycosidases and glycosyl transferases in glycoside and oligosaccharide synthesis. *Curr. Opin. Chem. Biol.* **2**, 98–111
77. Jacob, G. S., and Scudder, P. (1994) Glycosidases in structural analysis. *Methods Enzymol.* **230**, 280–299
78. Bojarová, P., and Křen, V. (2009) Glycosidases: a key to tailored carbohydrates. *Trends Biotechnol.* **27**, 199–209
79. Kightlinger, W., Warfel, K. F., Delisa, M. P., and Jewett, M. C. (2020) Synthetic glycobiology: parts, systems, and applications. *ACS Synth. Biol.* **9**, 1534–1562
80. Gotz, L., Abrahams, J. L., Mariethoz, J., Rudd, P. M., Karlsson, N. G., Packer, N. H., Campbell, M. P., and Lisacek, F. (2014) GlycoDigest: a tool for the targeted use of exoglycosidase digestions in glycan structure determination. *Bioinformatics* **30**, 3131–3133
81. Freeze, H. H., and Kranz, C. (2010) Endoglycosidase and glycoamidase release of N-linked glycans. *Curr. Protoc. Mol. Biol.* Chapter 17:Unit 17.13A
82. Stadlmann, J., Hoi, D. M., Taubenschmid, J., Mechtler, K., and Penninger, J. M. (2018) Analysis of PNGase F-resistant N-glycopeptides using SugarQb for proteome discoverer 2.1 reveals cryptic substrate specificities. *Proteomics* **18**, 1700436
83. Zielinska, D. F., Gnad, F., Wiśniewski, J. R., and Mann, M. (2010) Precision mapping of an *in vivo* N-glycoproteome reveals rigid topological and sequence constraints. *Cell* **141**, 897–907
84. Sun, S., Hu, Y., Ao, M., Shah, P., Chen, J., Yang, W., Jia, X., Tian, Y., Thomas, S., and Zhang, H. (2019) N-GlycositeAtlas: a database resource for mass spectrometry-based human N-linked glycoprotein and glycosylation site mapping. *Clin. Proteomics* **16**, 1–11
85. Wollscheid, B., Bausch-Fluck, D., Henderson, C., O'Brien, R., Bibel, M., Schiess, R., Aebersold, R., and Watts, J. D. (2009) Mass-spectrometric identification and relative quantification of N-linked cell surface glycoproteins. *Nat. Biotechnol.* **27**, 378–386
86. Fang, P., Wang, X.-J., Xue, Y., Liu, M.-Q., Zeng, W.-F., Zhang, Y., Zhang, L., Gao, X., Yan, G.-Q., Yao, J., Shen, H.-L., and Yang, P.-Y. (2016) In-depth mapping of the mouse brain N-glycoproteome reveals widespread N-glycosylation of diverse brain proteins. *Oncotarget* **7**, 38796–38809
87. Palmisano, G., Melo-Braga, M. N., Engholm-Keller, K., Parker, B. L., and Larsen, M. R. (2012) Chemical deamidation: a common pitfall in large-scale n-linked glycoproteomic mass spectrometry-based analyses. *J. Proteome Res.* **11**, 1949–1957
88. Keating, C. L., Kuhn, E., Bals, J., Cocco, A. R., Yousif, A. S., Matysiak, C., Sangesland, M., Ronsard, L., Smoot, M., Moreno, T. B., Okonkwo, V., Setliff, I., Georgiev, I., Balazs, A. B., Carr, S. A., *et al*. (2020) Spontaneous glycan reattachment following N-glycanase treatment of influenza and HIV vaccine antigens. *J. Proteome Res.* **19**, 733–743
89. Wells, L., Vosseller, K., Cole, R. N., Cronshaw, J. M., Matunis, M. J., and Hart, G. W. (2002) Mapping sites of O-GlcNAc modification using affinity tags for serine and threonine post-translational modifications. *Mol. Cell. Proteomics* **1**, 791–804
90. Boysen, A., Palmisano, G., Krogh, T. J., Duggin, I. G., Larsen, M. R., and Møller-Jensen, J. (2016) A novel mass spectrometric strategy "bEMAP" reveals extensive O-linked protein glycosylation in enterotoxigenic Escherichia coli. *Sci. Rep.* **6**, 32016
91. Cummings, R. D., and Pierce, J. M. (2014) The challenge and promise of glycomics. *Chem. Biol.* **21**, 1–15
92. Rojas-Macias, M. A., Mariethoz, J., Andersson, P., Jin, C., Venkatakrishnan, V., Aoki, N. P., Shinmachi, D., Ashwood, C., Madunic, K.,

Zhang, T., Miller, R. L., Horlacher, O., Struwe, W. B., Watanabe, Y., Okuda, S., *et al*. (2019) Towards a standardized bioinformatics infrastructure for N- and O-glycomics. *Nat. Commun.* **10**, 1–10
93. Ruhaak, L. R., Xu, G., Li, Q., Goonatilleke, E., and Lebrilla, C. B. (2018) Mass spectrometry approaches to glycomic and glycoproteomic analyses. *Chem. Rev.* **118**, 7886–7930
94. Li, Q., Xie, Y., Wong, M., Barboza, M., and Lebrilla, C. B. (2020) Comprehensive structural glycomic characterization of the glycocalyxes of cells and tissues. *Nat. Protoc.* **15**, 2668–2704
95. Chandler, K. B., and Costello, C. E. (2016) Glycomics and glycoproteomics of membrane proteins and cell-surface receptors: present trends and future opportunities. *Electrophoresis* **37**, 1407–1419
96. Hage, D. S., Anguizola, J. A., Li, R., Matsuda, R., Papastavros, E., Pfaunmiller, E., Sobansky, M., and Zheng, X. (2017). In: *Liquid Chromatography: Fundamentals and Instrumentation*, 2nd Ed, Elsevier, Amsterdam, Netherlands: 319–341
97. Lee, W. C., and Lee, K. H. (2004) Applications of affinity chromatography in proteomics. *Anal. Biochem.* **324**, 1–10
98. Sharon, N., and Lis, H. (2004) History of lectins: from hemagglutinins to biological recognition molecules. *Glycobiology* **14**, 483–491
99. Belardi, B., and Bertozzi, C. R. (2015) Chemical lectinology: tools for probing the ligands and dynamics of mammalian lectins in vivo. *Chem. Biol.* **22**, 983–993
100. Merkle, R. K., and Cummings, R. D. (1987) Lectin affinity chromatography of glycopeptides. *Methods Enzymol.* **138**, 232–259
101. Johannes, L., Jacob, R., and Leffler, H. (2018) Galectins at a glance. *J. Cell Sci.* **131**, jcs208884
102. Crocker, P. R., Paulson, J. C., and Varki, A. (2007) Siglecs and their roles in the immune system. *Nat. Rev. Immunol.* **7**, 255–266
103. Qiu, R., and Regnier, F. E. (2005) Use of multidimensional lectin affinity chromatography in differential glycoproteomics. *Anal. Chem.* **77**, 2802–2809
104. Ma, Z. Y., Skorobogatko, Y., and Vosseller, K. (2013) Tandem lectin weak affinity chromatography for glycoprotein enrichment. *Methods Mol. Biol.* **951**, 21–31
105. Xu, S. L., Chalkley, R. J., Maynard, J. C., Wang, W., Ni, W., Jiang, X., Shin, K., Cheng, L., Savage, D., Hühmer, A. F. R., Burlingame, A. L., and Wang, Z. Y. (2017) Proteomic analysis reveals O-GlcNAc modification on proteins with key regulatory functions in Arabidopsis. *Proc. Natl. Acad. Sci. U. S. A.* **114**, E1536–E1543
106. Kubota, Y., Fujioka, K., and Takekawa, M. (2017) WGA-based lectin affinity gel electrophoresis: a novel method for the detection of O-GlcNAc-modified proteins. *PLoS One* **12**, e0180714
107. Ma, J., and Hart, G. W. (2014) O-GlcNAc profiling: from proteins to proteomes. *Clin. Proteomics* **11**, 1–16
108. Nagel, A. K., Schilling, M., Comte-Walters, S., Berkaw, M. N., and Ball, L. E. (2013) Identification of O-linked N-acetylglucosamine (O-GlcNAc)-modified osteoblast proteins by electron transfer dissociation tandem mass spectrometry reveals proteins critical for bone formation. *Mol. Cell. Proteomics* **12**, 945–955
109. Skorobogatko, Y. V., Deuso, J., Adolf-Bergfoyle, J., Nowak, M. G., Gong, Y., Lippa, C. F., and Vosseller, K. (2011) Human Alzheimer's disease synaptic O-GlcNAc site mapping and iTRAQ expression proteomics with ion trap mass spectrometry. *Amino Acids* **40**, 765–779
110. Trinidad, J. C., Barkan, D. T., Gulledge, B. F., Thalhammer, A., Sali, A., Schoepfer, R., and Burlingame, A. L. (2012) Global identification and characterization of both O-GlcNAcylation and phosphorylation at the murine synapse. *Mol. Cell. Proteomics* **11**, 215–229
111. Trinidad, J. C., Schoepfer, R., Burlingame, A. L., and Medzihradszky, K. F. (2013) N- and O-glycosylation in the murine synaptosome. *Mol. Cell. Proteomics* **12**, 3474–3488
112. Medzihradszky, K. F., Kaasik, K., and Chalkley, R. J. (2015) Characterizing sialic acid variants at the glycopeptide level. *Anal. Chem.* **87**, 3064–3071
113. Pap, A., Klement, E., Hunyadi-Gulyas, E., Darula, Z., and Medzihradszky, K. F. (2018) Status report on the high-throughput characterization of complex intact O-glycopeptide mixtures. *J. Am. Soc. Mass Spectrom.* **29**, 1210–1220
114. Darula, Z., Pap, Á., and Medzihradszky, K. F. (2019) Extended sialylated O-glycan repertoire of human urinary glycoproteins discovered and characterized using electron-transfer/higher-energy collision dissociation. *J. Proteome Res.* **18**, 280–291
115. Pap, A., Tasnadi, E., Medzihradszky, K. F., and Darula, Z. (2020) Novel O-linked sialoglycan structures in human urinary glycoproteins. *Mol. Omics* **16**, 156–164
116. Wiśniewski, J. R., Zielinska, D. F., and Mann, M. (2011) Comparison of ultrafiltration units for proteomic and N-glycoproteomic analysis by the filter-aided sample preparation method. *Anal. Biochem.* **410**, 307–309
117. Boersema, P. J., Geiger, T., Wiśniewski, J. R., and Mann, M. (2013) Quantification of the N-glycosylated secretome by super-SILAC during breast cancer progression and in human blood samples. *Mol. Cell. Proteomics* **12**, 158–171
118. Deeb, S. J., Cox, J., Schmidt-Supprian, M., and Mann, M. (2014) N-linked glycosylation enrichment for in-depth cell surface proteomics of diffuse large B-cell lymphoma subtypes. *Mol. Cell. Proteomics* **13**, 240–251
119. Zeng, Z., Hincapie, M., Pitteri, S. J., Hanash, S., Schalkwijk, J., Hogan, J. M., Wang, H., and Hancock, W. S. (2011) A proteomics platform combining depletion, multi-lectin affinity chromatography (M-LAC), and isoelectric focusing to study the breast cancer proteome. *Anal. Chem.* **83**, 4845–4854
120. Wang, Y., Ao, X., Vuong, H., Konanur, M., Miller, F. R., Goodison, S., and Lubman, D. M. (2008) Membrane glycoproteins associated with breast tumor cell progression identified by a lectin affinity approach. *J. Proteome Res.* **7**, 4313–4325
121. Lee, L. Y., Hincapie, M., Packer, N., Baker, M. S., Hancock, W. S., and Fanayan, S. (2012) An optimized approach for enrichment of glycoproteins from cell culture lysates using native multi-lectin affinity chromatography. *J. Sep. Sci.* **35**, 2445–2452
122. Zhang, Y., Xie, X., Zhao, X., Tian, F., Lv, J., Ying, W., and Qian, X. (2018) Systems analysis of singly and multiply O-glycosylated peptides in the human serum glycoproteome via EThcD and HCD mass spectrometry. *J. Proteomics* **170**, 14–27
123. Darula, Z., Sarnyai, F., and Medzihradszky, K. F. (2016) O-glycosylation sites identified from mucin core-1 type glycopeptides from human serum. *Glycoconj. J.* **33**, 435–445
124. Durham, M., and Regnier, F. E. (2006) Targeted glycoproteomics: serial lectin affinity chromatography in the selection of O-glycosylation sites on proteins from the human blood proteome. *J. Chromatogr. A* **1132**, 165–173
125. King, S. L., Joshi, H. J., Schjoldager, K. T., Halim, A., Madsen, T. D., Dziegiel, M. H., Woetmann, A., Vakhrushev, S. Y., and Wandall, H. H. (2017) Characterizing the O-glycosylation landscape of human plasma, platelets, and endothelial cells. *Blood Adv.* **1**, 429–442
126. Totten, S. M., Kullolli, M., and Pitteri, S. J. (2017). In: *Methods in Molecular Biology*, Humana Press Inc, Clifton, NJ: 99–112
127. Totten, S. M., Adusumilli, R., Kullolli, M., Tanimoto, C., Brooks, J. D., Mallick, P., and Pitteri, S. J. (2018) Multi-lectin affinity chromatography and quantitative proteomic analysis reveal differential glycoform levels between prostate cancer and benign prostatic hyperplasia sera. *Sci. Rep.* **8**, 1–13
128. Vainauskas, S., Duke, R. M., McFarland, J., McClung, C., Ruse, C., and Taron, C. H. (2016) Profiling of core fucosylated N-glycans using a novel bacterial lectin that specifically recognizes α1,6 fucosylated chitobiose. *Sci. Rep.* **6**, 1–12
129. Lectins, H. O. F. (2017) Chapter 28 discovery and classification of glycan-binding proteins. In: *Essentials of Glycobiology*, 3rd Ed, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY: 1–12
130. Wu, D., Li, J., Struwe, W. B., and Robinson, C. V. (2019) Probing: N-glycoprotein microheterogeneity by lectin affinity purification-mass spectrometry analysis. *Chem. Sci.* **10**, 5146–5155
131. Gao, C., Hanes, M. S., Byrd-Leotis, L. A., Wei, M., Jia, N., Kardish, R. J., McKitrick, T. R., Steinhauer, D. A., and Cummings, R. D. (2019) Unique binding specificities of proteins toward isomeric asparagine-linked glycans. *Cell Chem. Biol.* **26**, 535–547.e4
132. Bonnardel, F., Mariethoz, J., Salentin, S., Robin, X., Schroeder, M., Perez, S., Lisacek, F. D. S., and Imberty, A. (2019) Unilectin3d, a database of carbohydrate binding proteins with curated information on 3D structures and interacting ligands. *Nucleic Acids Res.* **47**, D1236–D1244
133. Cummings, R. D., Schnaar, R. L., Esko, J. D., Drickamer, K., and Taylor, M. E. (2017) *Principles of Glycan Recognition*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

134. Pérez, S., Sarkar, A., Rivet, A., Drouillard, S., Breton, C., and Imberty, A. (2017). In: *A Practical Guide to Using Glycomics Databases*, Springer Japan, Tokyo, Japan: 133–161
135. Chandra, N. R., Kumar, N., Jeyakani, J., Singh, D. D., Gowda, S. B., and Prathima, M. N. (2006) Lectindb: a plant lectin database. *Glycobiology* **16**, 938–946
136. Hirabayashi, J., Tateno, H., Shikanai, T., Aoki-Kinoshita, K., and Narimatsu, H. (2015) The lectin frontier database (LfDB), and data generation based on frontal affinity chromatography. *Molecules* **20**, 951–973
137. Steentoft, C., Bennett, E. P., Schjoldager, K. T. B. G., Vakhrushev, S. Y., Wandall, H. H., and Clausen, H. (2014) Precision genome editing: a small revolution for glycobiology. *Glycobiology* **24**, 663–680
138. Clausen, H., Wandall, H. H., Steentoft, C., Stanley, P., and Schnaar, R. L. (2017) Chapter 56: glycosylation engineering. In: *Essentials Glycobiology*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY: 1–15
139. Steentoft, C., Vakhrushev, S. Y., Vester-Christensen, M. B., Schjoldager, K. T. B. G., Kong, Y., Bennett, E. P., Mandel, U., Wandall, H., Levery, S. B., and Clausen, H. (2011) Mining the O-glycoproteome using zinc-finger nuclease-glycoengineered SimpleCell lines. *Nat. Methods* **8**, 977–982
140. Steentoft, C., Vakhrushev, S. Y., Joshi, H. J., Kong, Y., Vester-Christensen, M. B., Schjoldager, K. T.-B. G., Lavrsen, K., Dabelsteen, S., Pedersen, N. B., Marcos-Silva, L., Gupta, R., Paul Bennett, E., Mandel, U., Brunak, S., Wandall, H. H., *et al*. (2013) Precision mapping of the human O-GalNAc glycoproteome through SimpleCell technology. *EMBO J.* **32**, 1478–1488
141. Vakhrushev, S. Y., Steentoft, C., Vester-Christensen, M. B., Bennett, E. P., Clausen, H., and Levery, S. B. (2013) Enhanced mass spectrometric mapping of the human GalNAc-type O-glycoproteome with simplecells. *Mol. Cell. Proteomics* **12**, 932–944
142. Narimatsu, Y., Joshi, H. J., Schjoldager, K. T., Hintze, J., Halim, A., Steentoft, C., Nason, R., Mandel, U., Bennett, E. P., Clausen, H., and Vakhrushev, S. Y. (2019) Exploring regulation of protein O-glycosylation in isogenic human HEK293 cells by differential O-glycoproteomics. *Mol. Cell. Proteomics* **18**, 1396–1409
143. Vester-Christensen, M. B., Halim, A., Joshi, H. J., Steentoft, C., Bennett, E. P., Levery, S. B., Vakhrushev, S. Y., and Clausen, H. (2013) Mining the O-mannose glycoproteome reveals cadherins as major O-mannosylated glycoproteins. *Proc. Natl. Acad. Sci. U. S. A.* **110**, 21018–21023
144. Neubert, P., Halim, A., Zauser, M., Essig, A., Joshi, H. J., Zatorska, E., Larsen, I. S. B., Loibl, M., Castells-Ballester, J., Aebi, M., Clausen, H., and Strahl, S. (2016) Mapping the O-Mannose glycoproteome in saccharomyces cerevisiae. *Mol. Cell. Proteomics* **15**, 1323–1337
145. Darula, Z., Sherman, J., and Medzihradszky, K. F. (2012) How to dig deeper? Improved enrichment methods for mucin core-1 type glycopeptides. *Mol. Cell. Proteomics* **11**, O111.016774
146. Bai, X., Li, D., Zhu, J., Guan, Y., Zhang, Q., and Chi, L. (2015) From individual proteins to proteomic samples: characterization of O-glycosylation sites in human chorionic gonadotropin and human-plasma proteins. *Anal. Bioanal. Chem.* **407**, 1857–1869
147. Darula, Z., and Medzihradszky, K. F. (2009) Affinity enrichment and characterization of mucin core-1 type glycopeptides from bovine serum. *Mol. Cell. Proteomics* **8**, 2515–2526
148. Mariappa, D., Selvan, N., Borodkin, V. S., Alonso, J., Ferenbach, A. T., Shepherd, C., Navratilova, I. H., and Van Aalten, D. M. F. (2015) A mutant O-GlcNAcase as a probe to reveal global dynamics of protein O-GlcNAcylation during Drosophila embryonic development. *Biochem. J.* **470**, 255–262
149. Yang, L., Wu, S., Cooper, J. C., Paul, M. K., Cummings, A. L., Eletr, Z. M., Ben-Arye, S. L., Padler-Karavani, V., Samli, K. N., and Woods, R. J. (2018) High-specificity affinity reagents for the detection of glycan sialylation. *Fed. Am. Soc. Exp. Biol.* https://doi.org/10.1096/fasebj.2018.32.1_supplement.544.16
150. Woods, R. J., and Yang, L. (2018) Glycan-specific analytical tools. *J. Comput. Chem.* **29**, Patent, number US9926612B2
151. Chen, M., Shi, X., Duke, R. M., Ruse, C. I., Dai, N., Taron, C. H., and Samuelson, J. C. (2017) An engineered high affinity Fbs1 carbohydrate binding protein for selective capture of N-glycans and N-glycopeptides. *Nat. Commun.* **8**, 15487
152. Ferreira, J. A., Daniel-da-Silva, A. L., Alves, R. M. P., Duarte, D., Vieira, I., Santos, L. L., Vitorino, R., and Amado, F. (2011) Synthesis and optimization of lectin functionalized nanoprobes for the selective recovery of glycoproteins from human body fluids. *Anal. Chem.* **83**, 7035–7043
153. Chen, Y. J., Dela Rosa, M. A. C., Chen, W. C., Chen, Y. J., Obena, R. P., Chang, C. H., Capangpangan, R. Y., Su, T. H., Chen, C. L., and Chen, P. J. (2017) One-pot two-nanoprobe assay uncovers targeted glycoprotein biosignature. *Anal. Chem.* **89**, 3973–3980
154. Lastovickova, M., Strouhalova, D., and Bobalova, J. (2020) Use of lectin-based affinity techniques in breast cancer glycoproteomics: a review. *J. Proteome Res.* **19**, 1885–1899
155. Sterner, E., Flanagan, N., and Gildersleeve, J. C. (2016) Perspectives on anti-glycan antibodies gleaned from development of a community resource database. *ACS Chem. Biol.* **11**, 1773–1783
156. Wang, Z., Pandey, A., and Hart, G. W. (2007) Dynamic interplay between O-linked N-acetylglucosaminylation and glycogen synthase kinase-3-dependent phosphorylation. *Mol. Cell. Proteomics* **6**, 1365–1379
157. Teo, C. F., Ingale, S., Wolfert, M. A., Elsayed, G. A., Nöt, L. G., Chatham, J. C., Wells, L., and Boons, G. J. (2010) Glycopeptide-specific monoclonal antibodies suggest new roles for O-GlcNAc. *Nat. Chem. Biol.* **6**, 338–343
158. Lavrsen, K., Madsen, C. B., Rasch, M. G., Woetmann, A., Ødum, N., Mandel, U., Clausen, H., Pedersen, A. E., and Wandall, H. H. (2013) Aberrantly glycosylated MUC1 is expressed on the surface of breast cancer cells and a target for antibody-dependent cell-mediated cytotoxicity. *Glycoconj. J.* **30**, 227–236
159. Sharma, P., Marada, V. V. V. R., Cai, Q., Kizerwetter, M., He, Y., Wolf, S. P., Schreiber, K., Clausen, H., Schreiber, H., and Kranz, D. M. (2020) Structure-guided engineering of the affinity and specificity of CARs against Tn-glycopeptides. *Proc. Natl. Acad. Sci. U. S. A.* **117**, 15148–15159
160. Matsumoto, Y., Kudelka, M. R., Hanes, M. S., Lehoux, S., Dutta, S., Jones, M. B., Stackhouse, K. A., Cervoni, G. E., Heimburg-Molinaro, J., Smith, D. F., Ju, T., Chaikof, E. L., and Cummings, R. D. (2020) Identification of Tn antigen O-GalNAc-expressing glycoproteins in human carcinomas using novel anti-Tn recombinant antibodies. *Glycobiology* **30**, 282–300
161. Naito, S., Takahashi, T., Onoda, J., Uemura, S., Ohyabu, N., Takemoto, H., Yamane, S., Fujii, I., Nishimura, S. I., and Numata, Y. (2017) Generation of novel anti-MUC1 monoclonal antibodies with designed carbohydrate specificities using MUC1 glycopeptide library. *ACS Omega* **2**, 7493–7505
162. Zhou, D., Xu, L., Huang, W., and Tonn, T. (2018) Epitopes of MUC1 tandem repeats in cancer as revealed by antibody crystallography: toward glycopeptide signature-guided therapy. *Molecules* **23**, 1326
163. Kim, K. H., Lee, S.-Y., Hwang, H., Lee, J. Y., Ji, E. S., An, H. J., Kim, J. Y., and Yoo, J. S. (2018) Direct monitoring of fucosylated glycopeptides of alpha-fetoprotein in human serum for early hepatocellular carcinoma by liquid chromatography-tandem mass spectrometry with immunoprecipitation. *Proteomics Clin. Appl.* **12**, e1800062
164. Kim, K. H., Park, G. W., Jeong, J. E., Ji, E. S., An, H. J., Kim, J. Y., and Yoo, J. S. (2019) Parallel reaction monitoring with multiplex immunoprecipitation of N-glycoproteins in human serum for detection of hepatocellular carcinoma. *Anal. Bioanal. Chem.* **411**, 3009–3019
165. McKitrick, T. R., Goth, C. K., Rosenberg, C. S., Nakahara, H., Heimburg-Molinaro, J., McQuillan, A. M., Falco, R., Rivers, N. J., Herrin, B. R., Cooper, M. D., and Cummings, R. D. (2020) Development of smart anti-glycan reagents using immunized lampreys. *Commun. Biol.* **3**, 1–12
166. McKitrick, T. R., Eris, D., Mondal, N., Aryal, R. P., McCurley, N., Heimburg-Molinaro, J., and Cummings, R. D. (2020) Antibodies from lampreys as smart anti-glycan reagents (SAGRs): perspectives on their specificity, structure, and glyco-genomics. *Biochemistry* **59**, 3111–3122
167. Riley, N. M., and Coon, J. J. (2016) Phosphoproteomics in the age of rapid and deep proteome profiling. *Anal. Chem.* **88**, 74–94
168. Low, T. Y., Mohtar, M. A., Lee, P. Y., Omar, N., Zhou, H., and Ye, M. (2020) Widening the bottleneck of phosphoproteomics: evolving strategies for phosphopeptide enrichment. *Mass Spectrom. Rev.* https://doi.org/10.1002/mas.21636
169. Larsen, M. R., Jensen, S. S., Jakobsen, L. A., and Heegaard, N. H. H. (2007) Exploring the sialiome using titanium dioxide chromatography and mass spectrometry. *Mol. Cell. Proteomics* **6**, 1778–1787

170. Palmisano, G., Lendal, S. E., Engholm-Keller, K., Leth-Larsen, R., Parker, B. L., and Larsen, M. R. (2010) Selective enrichment of sialic acid-containing glycopeptides using titanium dioxide chromatography with analysis by HILIC and mass spectrometry. *Nat. Protoc.* **5**, 1974–1982
171. Palmisano, G., Lendal, S. E., and Larsen, M. R. (2011) Titanium dioxide enrichment of sialic acid-containing glycopeptides. *Methods Mol. Biol.* **753**, 309–322
172. Melo-Braga, M. N., Ibáñez-Vea, M., Larsen, M. R., and Kulej, K. (2014) Comprehensive protocol to simultaneously study protein phosphorylation, acetylation, and N -linked sialylated glycosylation. *Methods Mol. Biol.* **1295**, 275–292
173. Melo-Braga, M. N., Schulz, M., Liu, Q., Swistowski, A., Palmisano, G., Engholm-Keller, K., Jakobsen, L., Zeng, X., and Larsen, M. R. (2014) Comprehensive quantitative comparison of the membrane proteome, phosphoproteome, and sialiome of human embryonic and neural stem cells. *Mol. Cell. Proteomics* **13**, 311–328
174. Lassen, P. S., Thygesen, C., Larsen, M. R., and Kempf, S. J. (2017) Understanding Alzheimer's disease by global quantification of protein phosphorylation and sialylated N-linked glycosylation profiles: a chance for new biomarkers in neuroproteomics? *J. Proteomics* **161**, 11–25
175. Boll, I., Jensen, P., Schwämmle, V., and Larsen, M. R. (2020) Depolarization-dependent induction of site-specific changes in sialylation on N -linked glycoproteins in rat nerve terminals. *Mol. Cell. Proteomics* **19**, 1418–1435
176. Klement, E., Raffai, T., and Medzihradszky, K. F. (2016) Immobilized metal affinity chromatography optimized for the analysis of extracellular phosphorylation. *Proteomics* **16**, 1858–1862
177. Zhu, J., Wang, F., Cheng, K., Dong, J., Sun, D., Chen, R., Wang, L., Ye, M., and Zou, H. (2013) A simple integrated system for rapid analysis of sialic-acid-containing *N* -glycopeptides from human serum. *Proteomics* **13**, 1306–1313
178. Zhang, H., Guo, T., Li, X., Datta, A., Park, J. E., Yang, J., Lim, S. K., Tam, J. P., and Sze, S. K. (2010) Simultaneous characterization of glyco- and phosphoproteomes of mouse brain membrane proteome with electrostatic repulsion hydrophilic interaction chromatography. *Mol. Cell. Proteomics* **9**, 635–647
179. Chen, R., Williamson, S., Fulton, K. M., Twine, S. M., and Li, J. (2019) Simultaneous analysis of phosphopeptides and intact glycopeptides from secretome with mode switchable solid phase extraction. *Anal. Methods* **11**, 5243–5249
180. Zou, X., Jie, J., and Yang, B. (2017) Single-step enrichment of N-glycopeptides and phosphopeptides with novel multifunctional Ti4+-immobilized dendritic polyglycerol coated chitosan nanomaterials. *Anal. Chem.* **89**, 7520–7526
181. Zheng, H., Jia, J., Li, Z., and Jia, Q. (2020) Bifunctional magnetic supramolecular-organic framework: a nanoprobe for simultaneous enrichment of glycosylated and phosphorylated peptides. *Anal. Chem.* **92**, 2680–2689
182. Sun, N., Wu, H., and Shen, X. (2020) Magnetic titanium dioxide nanomaterial modified with hydrophilic dicarboxylic ligand for effective enrichment and separation of phosphopeptides and glycopeptides. *Microchim. Acta* **187**, 195
183. Glover, M. S., Yu, Q., Chen, Z., Shi, X., Kent, K. C., and Li, L. (2018) Characterization of intact sialylated glycopeptides and phosphorylated glycopeptides from IMAC enriched samples by EThcD fragmentation: toward combining phosphoproteomics and glycoproteomics. *Int. J. Mass Spectrom.* **427**, 35–42
184. Huang, J., Dong, J., Shi, X., Chen, Z., Cui, Y., Liu, X., Ye, M., and Li, L. (2019) Dual-functional titanium(IV) immobilized metal affinity chromatography approach for enabling large-scale profiling of protein mannose-6-phosphate glycosylation and revealing its predominant substrates. *Anal. Chem.* **91**, 11589–11597
185. Hu, Y., Shah, P., Clark, D. J., Ao, M., and Zhang, H. (2018) Reanalysis of global proteomic and phosphoproteomic data identified a large number of glycopeptides. *Anal. Chem.* **90**, 8065–8071
186. Cho, K.-C., Chen, L., Hu, Y., Schnaubelt, M., and Zhang, H. (2019) Developing workflow for simultaneous analyses of phosphopeptides and glycopeptides. *ACS Chem. Biol.* **14**, 58–66
187. Zhou, Y., Lih, T. S. M., Yang, G., Chen, S. Y., Chen, L., Chan, D. W., Zhang, H., and Li, Q. K. (2020) An integrated workflow for global, glyco-, and phospho-proteomic analysis of tumor tissues. *Anal. Chem.* **92**, 1842–1849
188. Zheng, H., Guan, S., Wang, X., Zhao, J., Gao, M., and Zhang, X. (2020) Deconstruction of heterogeneity of size-dependent exosome subpopulations from human urine by profiling N-glycoproteomics and phosphoproteomics simultaneously. *Anal. Chem.* **92**, 9239–9246
189. Andaluz Aguilar, H., Iliuk, A. B., Chen, I. H., and Tao, W. A. (2020) Sequential phosphoproteomics and N-glycoproteomics of plasma-derived extracellular vesicles. *Nat. Protoc.* **15**, 161–180
190. Caval, T., Zhu, J., Tian, W., Remmelzwaal, S., Yang, Z., Clausen, H., and Heck, A. J. R. (2019) Targeted analysis of lysosomal directed proteins and their sites of mannose-6-phosphate modification. *Mol. Cell. Proteomics* **18**, 16–27
191. Diez, I. A., Govender, I., Naicker, P., Stoychev, S., Jordaan, J., and Jensen, O. N. (2021) Zirconium(IV)-IMAC for phosphopeptide enrichment in phosphoproteomics. *J. Proteome Res.* **20**, 452–462
192. Leney, A. C., El Atmioui, D., Wu, W., Ovaa, H., and Heck, A. J. R. (2017) Elucidating crosstalk mechanisms between phosphorylation and O-GlcNAcylation. *Proc. Natl. Acad. Sci. U. S. A.* **114**, E7255–E7261
193. Hägglund, P., Bunkenborg, J., Elortza, F., Jensen, O. N., and Roepstorff, P. (2004) A new strategy for identification of N-glycosylated proteins and unambiguous assignment of their glycosylation sites using HILIC enrichment and partial deglycosylation. *J. Proteome Res.* **3**, 556–566
194. Jandera, P. (2011) Stationary and mobile phases in hydrophilic interaction chromatography: a review. *Anal. Chim. Acta* **692**, 1–25
195. Jandera, P., and Janás, P. (2017) Recent advances in stationary phases and understanding of retention in hydrophilic interaction chromatography. A review. *Anal. Chim. Acta* **967**, 12–32
196. Sun, N., Wu, H., Chen, H., Shen, X., and Deng, C. (2019) Advances in hydrophilic nanomaterials for glycoproteomics. *Chem. Commun.* **55**, 10359–10375
197. Kozlik, P., Goldman, R., and Sanda, M. (2018) Hydrophilic interaction liquid chromatography in the separation of glycopeptides and their isomers. *Anal. Bioanal. Chem.* **410**, 5001–5008
198. Ikegami, T. (2019) Hydrophilic interaction chromatography for the analysis of biopharmaceutical drugs and therapeutic peptides: a review based on the separation characteristics of the hydrophilic interaction chromatography phases. *J. Sep. Sci.* **42**, 130–213
199. Domínguez-Vega, E., Tengattini, S., Peintner, C., van Angeren, J., Temporini, C., Haselberg, R., Massolini, G., and Somsen, G. W. (2018) High-resolution glycoform profiling of intact therapeutic proteins by hydrophilic interaction chromatography-mass spectrometry. *Talanta* **184**, 375–381
200. Zauner, G., Koeleman, C. A. M., Deelder, A. M., and Wuhrer, M. (2010) Protein glycosylation analysis by HILIC-LC-MS of proteinase K-generated *N* - and *O* -glycopeptides. *J. Sep. Sci.* **33**, 903–910
201. Qing, G., Yan, J., He, X., Li, X., and Liang, X. (2020) Recent advances in hydrophilic interaction liquid interaction chromatography materials for glycopeptide enrichment and glycan separation. *Trends Analyt. Chem.* **124**, 115570
202. Mysling, S., Palmisano, G., Hojrup, P., and Thaysen-Andersen, M. (2010) Utilizing ion-pairing hydrophilic interaction chromatography solid phase extraction for efficient glycopeptide enrichment in glycoproteomics. *Anal. Chem.* **82**, 5598–5609
203. Appelblad, P., Jonsson, T., Pontén, E., Viklund, C., and Jiang, W. (2008). In: *Merck SeQuant*, MerckSeQuant AB, former subsidiary of Merck KGaA group, Darmstadt, Germany: 1–28
204. Guo, Y. (2015) Recent progress in the fundamental understanding of hydrophilic interaction chromatography (HILIC). *Analyst* **140**, 6452–6466
205. Hemström, P., and Irgum, K. (2006) Hydrophilic interaction chromatography. *J. Sep. Sci.* **29**, 1784–1821
206. Alagesan, K., Khilji, S. K., and Kolarich, D. (2017) It is all about the solvent: on the importance of the mobile phase for ZIC-HILIC glycopeptide enrichment. *Anal. Bioanal. Chem.* **409**, 529–538
207. Betancourt, L. H., Espinosa, L. A., Ramos, Y., Bequet-Romero, M., Rodríguez, E. N., Sánchez, A., Marko-Varga, G., González, L. J., and Besada, V. (2019) Targeting the hydrophilic regions of recombinant proteins by MS via in-solution buffer-free trypsin digestion. *Eur. J. Mass Spectrom. (Chichester)* **26**, 230–237

208. Ahmad Izaham, A. R., and Scott, N. E. (2020) Open database searching enables the identification and comparison of bacterial glycoproteomes without defining glycan compositions prior to searching. *Mol. Cell. Proteomics* **19**, 1561–1574
209. Zhang, Y., Mao, Y., Zhao, W., Su, T., Zhong, Y., Fu, L., Zhu, J., Cheng, J., and Yang, H. (2020) Glyco-CPLL: an integrated method for in-depth and comprehensive N-glycoproteome profiling of human plasma. *J. Proteome Res.* **19**, 655–666
210. Shu, Q., Li, M., Shu, L., An, Z., Wang, J., Lv, H., Yang, M., Cai, T., Hu, T., Fu, Y., and Yang, F. (2020) Large-scale identification of N-linked intact glycopeptides in human serum using HILIC enrichment and spectral library search. *Mol. Cell. Proteomics* **19**, 672–689
211. Zhu, J., Lin, Y.-H., Dingess, K. A., Mank, M., Stahl, B., and Heck, A. J. R. (2020) Quantitative longitudinal inventory of the *N* -glycoproteome of human milk from a single donor reveals the highly variable repertoire and dynamic site-specific changes. *J. Proteome Res.* **19**, 1941–1952
212. Park, G. W., Kim, J. Y., Hwang, H., Lee, J. Y., Ahn, Y. H., Lee, H. K., Ji, E. S., Kim, K. H., Jeong, H. K., Yun, K. N., Kim, Y. S., Ko, J. H., An, H. J., Kim, J. H., Paik, Y. K., *et al*. (2016) Integrated GlycoProteome analyzer (I-GPA) for automated identification and quantitation of site-specific N-glycosylation. *Sci. Rep.* **6**, 1–12
213. Hwang, H., Jeong, H. K., Lee, H. K., Park, G. W., Lee, J. Y., Lee, S. Y., Kang, Y. M., An, H. J., Kang, J. G., Ko, J. H., Kim, J. Y., and Yoo, J. S. (2020) Machine learning classifies core and outer fucosylation of N-glycoproteins using mass spectrometry. *Sci. Rep.* **10**, 1–10
214. Yang, S., Wu, W. W., Shen, R. F., Bern, M., and Cipollo, J. (2018) Identification of sialic acid linkages on intact glycopeptides via differential chemical modification using IntactGIG-HILIC. *J. Am. Soc. Mass Spectrom.* **29**, 1273–1283
215. Wang, Y., Xu, F., Chen, Y., and Tian, Z. (2020) A quantitative N-glycoproteomics study of cell-surface N-glycoprotein markers of MCF-7/ADR cancer stem cells. *Anal. Bioanal. Chem.* **412**, 2423–2432
216. Xiao, K., and Tian, Z. (2019) GPSeeker enables quantitative structural N-glycoproteomics for site- and structure-specific characterization of differentially expressed N-glycosylation in hepatocellular carcinoma. *J. Proteome Res.* **18**, 2885–2895
217. Xue, B., Xiao, K., Wang, Y., and Tian, Z. (2020) Site- and structure-specific quantitative N-glycoproteomics study of differential N-glycosylation in MCF-7 cancer cells. *J. Proteomics* **212**, 103594
218. Xu, F., Wang, Y., Xiao, K., Hu, Y., Tian, Z., and Chen, Y. (2020) Quantitative site- and structure-specific N-glycoproteomics characterization of differential N-glycosylation in MCF-7/ADR cancer stem cells. *Clin. Proteomics* **17**, 3
219. Zhu, J., Chen, Z., Zhang, J., An, M., Wu, J., Yu, Q., Skilton, S. J., Bern, M., Ilker Sen, K., Li, L., and Lubman, D. M. (2019) Differential quantitative determination of site-specific intact N-glycopeptides in serum haptoglobin between hepatocellular carcinoma and cirrhosis using LC-EThcD-MS/MS. *J. Proteome Res.* **18**, 359–371
220. Lynn, K. S., Chen, C. C., Lih, T. M., Cheng, C. W., Su, W. C., Chang, C. H., Cheng, C. Y., Hsu, W. L., Chen, Y. J., and Sung, T. Y. (2015) MAGIC: an automated N-linked glycoprotein identification tool using a y1-ion pattern matching algorithm and in silico MS2 approach. *Anal. Chem.* **87**, 2466–2473
221. Chen, Z., Yu, Q., Hao, L., Liu, F., Johnson, J., Tian, Z., Kao, W. J., Xu, W., and Li, L. (2018) Site-specific characterization and quantitation of N-glycopeptides in PKM2 knockout breast cancer cells using DiLeu isobaric tags enabled by electron-transfer/higher-energy collision dissociation (EThcD). *Analyst* **143**, 2508–2519
222. Stadlmann, J., Taubenschmid, J., Wenzel, D., Gattinger, A., Dürnberger, G., Dusberger, F., Elling, U., Mach, L., Mechtler, K., and Penninger, J. M. (2017) Comparative glycoproteomics of stem cells identifies new players in ricin toxicity. *Nature* **549**, 538–542
223. Chen, R., Seebun, D., Ye, M., Zou, H., and Figeys, D. (2014) Site-specific characterization of cell membrane N-glycosylation with integrated hydrophilic interaction chromatography solid phase extraction and LC-MS/MS. *J. Proteomics* **103**, 194–203
224. Liu, M. Q., Zeng, W. F., Fang, P., Cao, W. Q., Liu, C., Yan, G. Q., Zhang, Y., Peng, C., Wu, J. Q., Zhang, X. J., Tu, H. J., Chi, H., Sun, R. X., Cao, Y., Dong, M. Q., *et al*. (2017) PGlyco 2.0 enables precision N-glycoproteomics with comprehensive quality control and one-step mass spectrometry for intact glycopeptide identification. *Nat. Commun.* **8**, 1–14
225. Parker, B. L., Thaysen-Andersen, M., Solis, N., Scott, N. E., Larsen, M. R., Graham, M. E., Packer, N. H., and Cordwell, S. J. (2013) Site-specific glycan-peptide analysis for determination of N-glycoproteome heterogeneity. *J. Proteome Res.* **12**, 5791–5800
226. Parker, B. L., Thaysen-Andersen, M., Fazakerley, D. J., Holliday, M., Packer, N. H., and James, D. E. (2016) Terminal galactosylation and sialylation switching on membrane glycoproteins upon TNF-alpha-induced insulin resistance in adipocytes. *Mol. Cell. Proteomics* **15**, 141–153
227. Fang, P., Xie, J. J., Sang, S., Zhang, L., Liu, M., Yang, L., Xu, Y., Yan, G., Yao, J., Gao, X., Qian, W., Wang, Z., Zhang, Y., Yang, P., and Shen, H. (2020) Multilayered N-glycoproteome profiling reveals highly heterogeneous and dysregulated protein N-glycosylation related to Alzheimer's disease. *Anal. Chem.* **92**, 867–874
228. Scott, N. E., Nothaft, H., Edwards, A. V. G., Labbate, M., Djordjevic, S. P., Larsen, M. R., Szymanski, C. M., and Cordwell, S. J. (2012) Modification of the Campylobacter jejuni N-linked glycan by EptC protein-mediated addition of phosphoethanolamine. *J. Biol. Chem.* **287**, 29384–29396
229. Scott, N. E., Parker, B. L., Connolly, A. M., Paulech, J., Edwards, A. V. G., Crossett, B., Falconer, L., Kolarich, D., Djordjevic, S. P., Hφjrup, P., Packer, N. H., Larsen, M. R., and Cordwell, S. J. (2011) Simultaneous glycan-peptide characterization using hydrophilic interaction chromatography and parallel fragmentation by CID, higher energy collisional dissociation, and electron transfer dissociation MS applied to the N-linked glycoproteome of Campylobacter jejuni. *Mol. Cell. Proteomics* **10**, M000031-MCP201
230. Lithgow, K. V., Scott, N. E., Iwashkiw, J. A., Thomson, E. L. S., Foster, L. J., Feldman, M. F., and Dennis, J. J. (2014) A general protein O-glycosylation system within the Burkholderia cepacia complex is involved in motility and virulence. *Mol. Microbiol.* **92**, 116–137
231. Iwashkiw, J. A., Seper, A., Weber, B. S., Scott, N. E., Vinogradov, E., Stratilo, C., Reiz, B., Cordwell, S. J., Whittal, R., Schild, S., and Feldman, M. F. (2012) Identification of a general O-linked protein glycosylation system in Acinetobacter baumannii and its role in virulence and biofilm formation. *PLoS Pathog.* **8**, e1002758
232. Zeng, W., Ford, K. L., Bacic, A., and Heazlewood, J. L. (2018) N-linked glycan micro-heterogeneity in glycoproteins of arabidopsis. *Mol. Cell. Proteomics* **17**, 413–421
233. Xue, Y., Xie, J., Fang, P., Yao, J., Yan, G., Shen, H., and Yang, P. (2018) Study on behaviors and performances of universal: N -glycopeptide enrichment methods. *Analyst* **143**, 1870–1880
234. Raudah, A., Izaham, A., Ang, C.-S., Nie, S., Bird, L. E., Williamson, N. A., and Scott, N. E. (2021) What are we missing by using hydrophilic enrichment? Improving bacterial glycoproteome 1 coverage using total proteome and FAIMS analysis. *J. Proteome Res.* **20**, 599–612
235. Neue, K., Mormann, M., Peter-Katalinić, J., and Pohlentz, G. (2011) Elucidation of glycoprotein structures by unspecific proteolysis and direct nanoESI mass spectrometric analysis of ZIC-HILIC-enriched glycopeptides. *J. Proteome Res.* **10**, 2248–2260
236. Wang, S., Qin, H., Mao, J., Fang, Z., Chen, Y., Zhang, X., Hu, L., and Ye, M. (2020) Profiling of endogenous intact N-linked and O-linked glycopeptides from human serum by using an integrated platform. *J. Proteome Res.* **19**, 1423–1434
237. Zhao, X., Zheng, S., Li, Y., Huang, J., Zhang, W., Xie, Y., Qin, W., and Qian, X. (2019) An integrated mass spectroscopy data processing strategy for fast identification, in-depth, and reproducible quantification of protein O-glycosylation in a large cohort of human urine samples. *Anal. Chem.* **92**, 690–698
238. Hoffmann, M., Marx, K., Reichl, U., Wuhrer, M., and Rapp, E. (2016) Site-specific O-glycosylation analysis of human blood plasma proteins. *Mol. Cell. Proteomics* **15**, 624–641
239. Shen, B., Zhang, W., Shi, Z., Tian, F., Deng, Y., Sun, C., Wang, G., Qin, W., and Qian, X. (2017) A novel strategy for global mapping of O-GlcNAc proteins and peptides using selective enzymatic deglycosylation, HILIC enrichment and mass spectrometry identification. *Talanta* **169**, 195–202
240. You, X., Qin, H., Mao, J., Tian, Y., Dong, M., Guo, Z., Liang, X., Wang, L., Jin, Y., and Ye, M. (2018) Highly efficient identification of O-GalNAc glycosylation by an acid-assisted glycoform simplification approach. *Proteomics* **18**, 1800042

241. Ohta, Y., Kameda, K., Matsumoto, M., and Kawasaki, N. (2017) Rapid glycopeptide enrichment using cellulose hydrophilic interaction/reversed-phase StageTips. *Mass Spectrom.* **6**, A0061
242. Zhou, Y., Xu, Y., Zhang, C., Emmer, Å., and Zheng, H. (2020) Amino acid-functionalized two-dimensional hollow cobalt sulfide nanoleaves for the highly selective enrichment of N-linked glycopeptides. *Anal. Chem.* **92**, 2151–2158
243. Pu, C., Zhao, H., Hong, Y., Zhan, Q., and Lan, M. (2020) Facile preparation of hydrophilic mesoporous metal-organic framework via synergistic etching and surface functionalization for glycopeptides analysis. *Anal. Chem.* **92**, 1940–1947
244. Yang, S.-S., Wang, C., Yu, X., Shang, W., Da Yong Chen, D., and Gu, Z.-Y. (2020) A hydrophilic two-dimensional titanium-based metal-organic framework nanosheets for specific enrichment of glycopeptides. *Anal. Chim. Acta* **1119**, 60–67
245. Wu, Y., Sun, N., and Deng, C. (2020) Construction of magnetic covalent organic frameworks with inherent hydrophilicity for efficiently enriching endogenous glycopeptides in human saliva. *ACS Appl. Mater. Inter.* **12**, 9814–9823
246. Saleem, S., Sajid, M. S., Hussain, D., Jabeen, F., Najam-ul-Haq, M., and Saeed, A. (2020) Boronic acid functionalized MOFs as HILIC material for N-linked glycopeptide enrichment. *Anal. Bioanal. Chem.* **412**, 1509–1520
247. Li, X., Zhang, H., Zhang, N., Ma, S., Ou, J., and Ye, M. (2019) One-step preparation of zwitterionic-rich hydrophilic hydrothermal carbonaceous materials for enrichment of N-glycopeptides. *ACS Sustain. Chem. Eng.* **7**, 11511–11520
248. Sajid, M. S., Jovcevski, B., Pukala, T. L., Jabeen, F., and Najam-Ul-Haq, M. (2020) Fabrication of piperazine functionalized polymeric monolithic tip for rapid enrichment of glycopeptides/glycans. *Anal. Chem.* **92**, 683–689
249. Lu, Q., Chen, C., Xiong, Y., Li, G., Zhang, X., Zhang, Y., Wang, D., Zhu, Z., Li, X., Qing, G., Sun, T., and Liang, X. (2020) High-efficiency phosphopeptide and glycopeptide simultaneous enrichment by hydrogen bond–based bifunctional smart polymer. *Anal. Chem.* **92**, 6269–6277
250. Alpert, A. J. (2008) Electrostatic repulsion hydrophilic interaction chromatography for isocratic separation of charged solutes and selective isolation of phosphopeptides. *Anal. Chem.* **80**, 62–76
251. Alpert, A. J., Hudecz, O., and Mechtler, K. (2015) Anion-exchange chromatography of phosphopeptides: weak anion exchange versus strong anion exchange and anion-exchange chromatography versus electrostatic repulsion-hydrophilic interaction chromatography. *Anal. Chem.* **87**, 4704–4711
252. Cao, L., Yu, L., Guo, Z., Li, X., Xue, X., and Liang, X. (2013) Application of a strong anion exchange material in electrostatic repulsion–hydrophilic interaction chromatography for selective enrichment of glycopeptides. *J. Chromatogr. A* **1299**, 18–24
253. Totten, S. M., Feasley, C. L., Bermudez, A., and Pitteri, S. J. (2017) Parallel comparison of N-linked glycopeptide enrichment techniques reveals extensive glycoproteomic analysis of plasma enabled by SAX-ERLIC. *J. Proteome Res.* **16**, 1249–1260
254. Zacharias, L. G., Hartmann, A. K., Song, E., Zhao, J., Zhu, R., Mirzaei, P., and Mechref, Y. (2016) HILIC and ERLIC enrichment of glycopeptides derived from breast and brain cancer cells. *J. Proteome Res.* **15**, 3624–3634
255. Cui, Y., Yang, K., Tabang, D. N., Huang, J., Tang, W., and Li, L. (2019) Finding the sweet spot in ERLIC mobile phase for simultaneous enrichment of N-glyco and phosphopeptides. *J. Am. Soc. Mass Spectrom.* **30**, 2491–2501
256. Yang, G., Hu, Y., Sun, S., Ouyang, C., Yang, W., Wang, Q., Betenbaugh, M., and Zhang, H. (2018) Comprehensive glycoproteomic analysis of Chinese hamster ovary cells. *Anal. Chem.* **90**, 14294–14302
257. Zhou, J., Yang, W., Hu, Y., Höti, N., Liu, Y., Shah, P., Sun, S., Clark, D., Thomas, S., and Zhang, H. (2017) Site-specific fucosylation analysis identifying glycoproteins associated with aggressive prostate cancer cell lines using tandem affinity enrichments of intact glycopeptides followed by mass spectrometry. *Anal. Chem.* **89**, 7623–7630
258. Yang, W., Shah, P., Hu, Y., Toghi Eshghi, S., Sun, S., Liu, Y., and Zhang, H. (2017) Comparison of enrichment methods for intact N- and O-linked glycopeptides using strong anion exchange and hydrophilic interaction liquid chromatography. *Anal. Chem.* **89**, 11193–11197
259. Yang, W., Song, A., Ao, M., Xu, Y., and Zhang, H. (2020) Large-scale site-specific mapping of the O-GalNAc glycoproteome. *Nat. Protoc.* **15**, 2589–2610
260. Yang, G., Höti, N., Chen, S. Y., Zhou, Y., Wang, Q., Betenbaugh, M., and Zhang, H. (2020) One-step enrichment of intact glycopeptides from glycoengineered Chinese hamster ovary cells. *Front. Chem.* **8**, 240
261. West, C., Elfakir, C., and Lafosse, M. (2010) Porous graphitic carbon: a versatile stationary phase for liquid chromatography. *J. Chromatogr. A* **1217**, 3201–3216
262. Bapiro, T. E., Richards, F. M., and Jodrell, D. I. (2016) Understanding the complexity of porous graphitic carbon (PGC) chromatography: modulation of mobile-stationary phase interactions overcomes loss of retention and reduces variability. *Anal. Chem.* **88**, 6190–6194
263. Cao, L., Qu, Y., Zhang, Z., Wang, Z., Prytkova, I., and Wu, S. (2016) Intact glycopeptide characterization using mass spectrometry. *Expert Rev. Proteomics* **13**, 513–522
264. Kolarich, D., Windwarder, M., Alagesan, K., and Altmann, F. (2015). In: *Glyco-Engineering: Methods and Protocols*, Springer New York, New York, NY: 427–435
265. Wei, J., Tang, Y., Bai, Y., Zaia, J., Costello, C. E., Hong, P., and Lin, C. (2020) Toward automatic and comprehensive glycan characterization by online PGC-LC-EED MS/MS. *Anal. Chem.* **92**, 782–791
266. Alagesan, K., Everest-Dass, A., and Kolarich, D. (2018). In: *Advances in Experimental Medicine and Biology*, Springer New York LLC, Singapore: 77–99
267. Melmer, M., Stangler, T., Premstaller, A., and Lindner, W. (2011) Comparison of hydrophilic-interaction, reversed-phase and porous graphitic carbon chromatography for glycan analysis. *J. Chromatogr. A* **1218**, 118–123
268. Ashwood, C., Pratt, B., Maclean, B. X., Gundry, R. L., and Packer, N. H. (2019) Standardization of PGC-LC-MS-based glycomics for sample specific glycotyping. *Analyst* **144**, 3601–3612
269. Ashwood, C., Waas, M., Weerasekera, R., and Gundry, R. L. (2020) Reference glycan structure libraries of primary human cardiomyocytes and pluripotent stem cell-derived cardiomyocytes reveal cell-type and culture stage-specific glycan phenotypes. *J. Mol. Cell. Cardiol.* **139**, 33–46
270. Ashwood, C., Lin, C.-H., Thaysen-Andersen, M., and Packer, N. H. (2018) Discrimination of isomers of released N-and O-glycans using diagnostic product ions in negative ion. *J. Am. Soc. Mass Spectrom.* **29**, 1194–1209
271. An, H. J., Peavy, T. R., Hedrick, J. L., and Lebrilla, C. B. (2003) Determination of N-glycosylation sites and site heterogeneity in glycoproteins. *Anal. Chem.* **75**, 5628–5637
272. Seo, Y., Oh, M. J., Park, J. Y., Ko, J. K., Kim, J. Y., and An, H. J. (2019) Comprehensive characterization of biotherapeutics by selective capturing of highly acidic glycans using stepwise PGC-SPE and LC/MS/MS. *Anal. Chem.* **91**, 6064–6071
273. Cao, L., Tolić, N., Qu, Y., Meng, D., Zhao, R., Zhang, Q., Moore, R. J., Zink, E. M., Lipton, M. S., Paša-Tolić, L., and Wu, S. (2014) Characterization of intact N- and O-linked glycopeptides using higher energy collisional dissociation. *Anal. Biochem.* **452**, 96–102
274. Chen, R., Stupak, J., Williamson, S., Twine, S. M., and Li, J. (2019) Online porous graphic carbon chromatography coupled with tandem mass spectrometry for post-translational modification analysis. *Rapid Commun. Mass Spectrom.* **33**, 1240–1247
275. Hua, S., Hu, C. Y., Kim, B. J., Totten, S. M., Oh, M. J., Yun, N., Nwosu, C. C., Yoo, J. S., Lebrilla, C. B., and An, H. J. (2013) Glyco-analytical multispecific proteolysis (Glyco-AMP): a simple method for detailed and quantitative glycoproteomic characterization. *J. Proteome Res.* **12**, 4414–4423
276. Nwosu, C. C., Seipert, R. R., Strum, J. S., Hua, S. S., An, H. J., Zivkovic, A. M., German, B. J., and Lebrilla, C. B. (2011) Simultaneous and extensive site-specific N- and O-glycosylation analysis in protein mixtures. *J. Proteome Res.* **10**, 2612–2624
277. Alley, W. R., Mechref, Y., and Novotny, M. V. (2009) Use of activated graphitized carbon chips for liquid chromatography/mass spectrometric and tandem mass spectrometric analysis of tryptic glycopeptides. *Rapid Commun. Mass Spectrom.* **23**, 495–505
278. Froehlich, J. W., Barboza, M., Chu, C., Lerno, L. A., Clowers, B. H., Zivkovic, A. M., German, J. B., and Lebrilla, C. B. (2011) Nano-LC-MS/MS

of glycopeptides produced by nonspecific proteolysis enables rapid and extensive site-specific glycosylation determination. *Anal. Chem.* **83**, 5541–5547
279. Huang, J., Guerrero, A., Parker, E., Strum, J. S., Smilowitz, J. T., German, J. B., and Lebrilla, C. B. (2015) Site-specific glycosylation of secretory immunoglobulin a from human colostrum. *J. Proteome Res.* **14**, 1335–1349
280. Dodds, E. D., Seipert, R. R., Clowers, B. H., German, J. B., and Lebrilla, C. B. (2009) Analytical performance of immobilized pronase for glycopeptide footprinting and implications for surpassing reductionist glycoproteomics. *J. Proteome Res.* **8**, 502–512
281. Stavenhagen, K., Hinneburg, H., Kolarich, D., and Wuhrer, M. (2017). In: *Methods in Molecular Biology*, Humana Press Inc, New York, NY: 109–119
282. Lewandrowski, U., and Sickmann, A. (2010) Online dual gradient reversed-phase/porous graphitized carbon nanoHPLC for proteomic applications. *Anal. Chem.* **82**, 5391–5396
283. Zhu, R., Huang, Y., Zhao, J., Zhong, J., and Mechref, Y. (2020) Isomeric separation of N-glycopeptides derived from glycoproteins by porous graphitic carbon (PGC) LC-MS/MS. *Anal. Chem.* **92**, 9556–9565
284. Ji, E. S., Lee, H. K., Park, G. W., Kim, K. H., Kim, J. Y., and Yoo, J. S. (2019) Isomer separation of sialylated O- and N-linked glycopeptides using reversed-phase LC–MS/MS at high temperature. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **1110–1111**, 101–107
285. Mertz, J. L., Toonstra, C., and Zhang, H. (2019). In: *Mass Spectrometry-Based Chemical Proteomics*, Wiley, Hoboken, NJ: 293–316
286. Haan, N. de, Yang, S., Cipollo, J., and Wuhrer, M. (2020) Glycomics studies using sialic acid derivatization and mass spectrometry. *Nat. Rev. Chem.* **2020**, 1–14
287. Zhang, H., Li, X. J., Martin, D. B., and Aebersold, R. (2003) Identification and quantification of N-linked glycoproteins using hydrazide chemistry, stable isotope labeling and mass spectrometry. *Nat. Biotechnol.* **21**, 660–666
288. Yang, S., Rubin, A., Eshghi, S. T., and Zhang, H. (2016) Chemoenzymatic method for glycomics: isolation, identification, and quantitation. *Proteomics* **16**, 241–256
289. Chen, J., Shah, P., and Zhang, H. (2013) Solid phase extraction of N-linked glycopeptides using hydrazide tip. *Anal. Chem.* **85**, 10670–10674
290. Yang, S., Zhang, L., Thomas, S., Hu, Y., Li, S., Cipollo, J., and Zhang, H. (2017) Modification of sialic acids on solid phase: accurate characterization of protein sialylation. *Anal. Chem.* **89**, 6330–6335
291. Yang, S., Clark, D., Liu, Y., Li, S., and Zhang, H. (2017) High-throughput analysis of N-glycans using AutoTip via glycoprotein immobilization. *Sci. Rep.* **7**, 1–11
292. Yang, S., Höti, N., Yang, W., Liu, Y., Chen, L., Li, S., and Zhang, H. (2017) Simultaneous analyses of N-linked and O-linked glycans of ovarian cancer cells using solid-phase chemoenzymatic method. *Clin. Proteomics* **14**, 3
293. Yang, S., and Zhang, H. (2014) Glycomic analysis of glycans released from glycoproteins using chemical immobilization and mass spectrometry. *Curr. Protoc. Chem. Biol.* **6**, 191–208
294. Zhang, Z., Sun, D., Cong, Y., Mao, J., Huang, J., Qin, H., Liu, J., Huang, G., Wang, L., Ye, M., and Zou, H. (2015) Amine chemistry method for selective enrichment of N-linked glycopeptides for glycoproteomics analysis. *J. Proteome Res.* **14**, 3892–3899
295. Huang, J., Wan, H., Yao, Y., Li, J., Cheng, K., Mao, J., Chen, J., Wang, Y., Qin, H., Zhang, W., Ye, M., and Zou, H. (2015) Highly efficient release of glycopeptides from hydrazide beads by hydroxylamine assisted PNGase F deglycosylation for N-glycoproteome analysis. *Anal. Chem.* **87**, 10199–10204
296. Shah, P., Wang, X., Yang, W., Eshghi, S. T., Sun, S., Hoti, N., Chen, L., Yang, S., Pasay, J., Rubin, A., and Zhang, H. (2015) Integrated proteomic and glycoproteomic analyses of prostate cancer cells reveal glycoprotein alteration in protein abundance and glycosylation. *Mol. Cell. Proteomics* **14**, 2753–2763
297. Zarif, J. C., Yang, W., Hernandez, J. R., Zhang, H., and Pienta, K. J. (2017) The identification of macrophage-enriched glycoproteins using glycoproteomics. *Mol. Cell. Proteomics* **16**, 1029–1037
298. Sun, S., Shah, P., Eshghi, S. T., Yang, W., Trikannad, N., Yang, S., Chen, L., Aiyetan, P., Höti, N., Zhang, Z., Chan, D. W., and Zhang, H. (2016) Comprehensive analysis of protein glycosylation by solid-phase extraction of N-linked glycans and glycosite-containing peptides. *Nat. Biotechnol.* **34**, 84–88
299. Sun, S., and Zhang, H. (2015) Identification and validation of atypical N-glycosylation sites. *Anal. Chem.* **87**, 11948–11951
300. Mertz, J. L., Sun, S., Yin, B., Betenbaugh, M. J., Yarema, K. J., and Zhang, H. (2020) Comparison of three glycoproteomic methods for the analysis of CHO cells treated with 1,3,4-O-Bu3ManNAc. *Bioeng.* **7**, 144
301. Yang, W., Ao, M., Hu, Y., Li, Q. K., and Zhang, H. (2018) Mapping the O-glycoproteome using site-specific extraction of O-linked glycopeptides (EXoO). *Mol. Syst. Biol.* **14**, e8486
302. Yang, S., Onigman, P., Wu, W. W., Sjogren, J., Nyhlen, H., Shen, R. F., and Cipollo, J. (2018) Deciphering protein O-glycosylation: solid-phase chemoenzymatic cleavage and enrichment. *Anal. Chem.* **90**, 8261–8269
303. Yang, S., Wu, W., Shen, R., Sjogren, J., Parsons, L., and Cipollo, J. (2020) Optimization of O-GIG for O-glycopeptide characterization with sialic acid linkage determination. *Anal. Chem.* **92**, 10946–10951
304. Lood, R., Nordgren, M., Leo, F., Björk, S., Mejàre, M., Nyhlén, H., and Olsson, F. (2018). In: *66TH ASMS Conference on Mass Spectrometry and Allied Topics*, American Society for Mass Spectrometry, USA
305. Riley, N., Malaker, S., and Bertozzi, C. (2020) Electron-Based Dissociation Is Needed for O-Glycopeptides Derived from OpeRATOR Proteolysis. *Anal. Chem.* **92**, 14878–14884
306. Li, Y., Qin, H., and Ye, M. (2020) An overview on enrichment methods for cell surface proteome profiling. *J. Sep. Sci.* **43**, 292–312
307. Suttapitugsakul, S., Ulmer, L. D., Jiang, C., Sun, F., and Wu, R. (2019) Surface glycoproteomic analysis reveals that both unique and differential expression of surface glycoproteins determine the cell type. *Anal. Chem.* **91**, 6934–6942
308. Nilsson, J., Rüetschi, U., Halim, A., Hesse, C., Carlsohn, E., Brinkmalm, G., and Larson, G. (2009) Enrichment of glycopeptides for glycan structure and attachment site identification. *Nat. Methods* **6**, 809–811
309. Nilsson, J., and Larson, G. (2013) Sialic acid capture-and-release and LC-MSn analysis of glycopeptides. *Methods Mol. Biol.* **951**, 79–100
310. Bayer, E. A., Ben-Hur, H., and Wilchek, M. (1988) Biocytin hydrazide-A selective label for sialic acids, galactose, and other sugars in glycoconjugates using avidin-biotin technology. *Anal. Biochem.* **170**, 271–281
311. van Oostrum, M., Müller, M., Klein, F., Bruderer, R., Zhang, H., Pedrioli, P. G. A., Reiter, L., Tsapogas, P., Rolink, A., and Wollscheid, B. (2019) Classification of mouse B cell types using surfaceome proteotype maps. *Nat. Commun.* **10**, 1–9
312. Bausch-Fluck, D., Milani, E. S., and Wollscheid, B. (2019) Surfaceome nanoscale organization and extracellular interaction networks. *Curr. Opin. Chem. Biol.* **48**, 26–33
313. Leung, K. K., Nguyen, A., Shi, T., Tang, L., Ni, X., Escoubet, L., MacBeth, K. J., DiMartino, J., and Wells, J. A. (2019) Multiomics of azacitidine-treated AML cells reveals variable and convergent targets that remodel the cell-surface proteome. *Proc. Natl. Acad. Sci. U. S. A.* **116**, 695–700
314. Wei, J., Leung, K., Truillet, C., Ruggero, D., Wells, J. A., and Evans, M. J. (2020) Profiling the surfaceome identifies therapeutic targets for cells with hyperactive mTORC1 signaling. *Mol. Cell. Proteomics* **19**, 297–307
315. Hofmann, A., Thiesler, T., Gerrits, B., Behnke, S., Sobotzki, N., Omasits, U., Bausch-Fluck, D., Bock, T., Aebersold, R., Moch, H., Tinguely, M., and Wollscheid, B. (2015) Surfaceome of classical Hodgkin and non-Hodgkin lymphoma. *Proteomics Clin. Appl.* **9**, 661–670
316. Bausch-Fluck, D., Hofmann, A., Bock, T., Frei, A. P., Cerciello, F., Jacobs, A., Moest, H., Omasits, U., Gundry, R. L., Yoon, C., Schiess, R., Schmidt, A., Mirkowska, P., Härtlová, A., Van Eyk, J. E., *et al*. (2015) A mass spectrometric-derived cell surface protein atlas. *PLoS One* **10**, e0121314
317. DeVeale, B., Bausch-Fluck, D., Seaberg, R., Runciman, S., Akbarian, V., Karpowicz, P., Yoon, C., Song, H., Leeder, R., Zandstra, P. W., Wollscheid, B., and van der Kooy, D. (2014) Surfaceome profiling reveals regulators of neural stem cell function. *Stem Cells* **32**, 258–268
318. Hofmann, A., Bausch-Fluck, D., and Wollscheid, B. (2013) CSC technology: selective labeling of glycoproteins by mild oxidation to phenotype cells. *Methods Mol. Biol.* **951**, 33–43
319. Gundry, R. L., Riordon, D. R., Tarasova, Y., Chuppa, S., Bhattacharya, S., Juhasz, O., Wiedemeier, O., Milanovich, S., Noto, F. K., Tchernyshyov, I., Raginski, K., Bausch-Fluck, D., Tae, H. J., Marshall, S., Duncan, S. A., *et al*. (2012) A cell surfaceome map for immunophenotyping and sorting pluripotent stem cells. *Mol. Cell. Proteomics* **11**, 303–316

320. Leung, K. K., Wilson, G. M., Kirkemo, L. L., Riley, N. M., Coon, J. J., and Wells, J. A. (2020) Broad and thematic remodeling of the surfaceome and glycoproteome on isogenic cells transformed with driving proliferative oncogenes. *Proc. Natl. Acad. Sci. U. S. A.* **117**, 7764–7775
321. Mallanna, S. K., Waas, M., Duncan, S. A., and Gundry, R. L. (2017) *N*-glycoprotein surfaceome of human induced pluripotent stem cell derived hepatic endoderm. *Proteomics* **17**, 1600397
322. Haverland, N. A., Waas, M., Ntai, I., Keppel, T., Gundry, R. L., and Kelleher, N. L. (2017) Cell surface proteomics of N-linked glycoproteins for typing of human lymphocytes. *Proteomics* **17**, 1700156
323. Zeng, Y., Ramya, T. N. C., Dirksen, A., Dawson, P. E., and Paulson, J. C. (2009) High-efficiency labeling of sialylated glycoproteins on living cells. *Nat. Methods* **6**, 207–209
324. Kalxdorf, M., Gade, S., Eberl, H. C., and Bantscheff, M. (2017) Monitoring cell-surface N-glycoproteome dynamics by quantitative proteomics reveals mechanistic insights into macrophage differentiation. *Mol. Cell. Proteomics* **16**, 770–785
325. Cai, Y., Zhang, Y., Yuan, W., Yao, J., Yan, G., and Lu, H. (2020) A thiazolidine formation-based approach for ultrafast and highly efficient solid-phase extraction of N-glycoproteome. *Anal. Chim. Acta* **1100**, 174–181
326. Frei, A. P., Jeon, O. Y., Kilcher, S., Moest, H., Henning, L. M., Jost, C., Plückthun, A., Mercer, J., Aebersold, R., Carreira, E. M., and Wollscheid, B. (2012) Direct identification of ligand-receptor interactions on living cells and tissues. *Nat. Biotechnol.* **30**, 997–1001
327. Frei, A. P., Moest, H., Novy, K., and Wollscheid, B. (2013) Ligand-based receptor identification on living cells and tissues using TRICEPS. *Nat. Protoc.* **8**, 1321–1336
328. Tremblay, T. L., and Hill, J. J. (2017) Biotin-transfer from a trifunctional crosslinker for identification of cell surface receptors of soluble protein ligands. *Sci. Rep.* **7**, 1–9
329. Sobotzki, N., Schafroth, M. A., Rudnicka, A., Koetemann, A., Marty, F., Goetze, S., Yamauchi, Y., Carreira, E. M., and Wollscheid, B. (2018) HATRIC-based identification of receptors for orphan ligands. *Nat. Commun.* **9**, 1–8
330. Ramya, T. N. C., Weerapana, E., Cravatt, B. F., and Paulson, J. C. (2013) Glycoproteomics enabled by tagging sialic acid-or galactose-terminated glycans. *Glycobiology* **23**, 211–221
331. Sun, F., Suttapitugsakul, S., and Wu, R. (2019) Enzymatic tagging of glycoproteins on the cell surface for their global and site-specific analysis with mass spectrometry. *Anal. Chem.* **91**, 4195–4203
332. Zheng, J., Xiao, H., and Wu, R. (2017) Specific identification of glycoproteins bearing the Tn antigen in human cells. *Angew. Chem. Int. Ed. Engl.* **56**, 7107–7111
333. McCombs, J. E., and Kohler, J. J. (2016) Pneumococcal neuraminidase substrates identified through comparative proteomics enabled by chemoselective labeling. *Bioconjug. Chem.* **27**, 1013–1022
334. Taga, Y., Kusubata, M., Ogawa-Goto, K., and Hattori, S. (2012) Development of a novel method for analyzing collagen O-glycosylations by hydrazide chemistry. *Mol. Cell. Proteomics* **11**, M111.010397
335. You, X., Yao, Y., Mao, J., Qin, H., Liang, X., Wang, L., and Ye, M. (2018) Chemoenzymatic approach for the proteomics analysis of mucin-type core-1 O-glycosylation in human serum. *Anal. Chem.* **90**, 12714–12722
336. Rannes, J. B., Ioannou, A., Willies, S. C., Grogan, G., Behrens, C., Flitsch, S. L., and Turner, N. J. (2011) Glycoprotein labeling using engineered variants of galactose oxidase obtained by directed evolution. *J. Am. Chem. Soc.* **133**, 8436–8439
337. Klement, E., Lipinszki, Z., Kupihár, Z., Udvardy, A., and Medzihradszky, K. F. (2010) Enrichment of O-GlcNAc modified proteins by the periodate oxidation-hydrazide resin capture approach. *J. Proteome Res.* **9**, 2200–2206
338. Huang, J., Qin, H., Sun, Z., Huang, G., Mao, J., Cheng, K., Zhang, Z., Wan, H., Yao, Y., Dong, J., Zhu, J., Wang, F., Ye, M., and Zou, H. (2015) A peptide N-terminal protection strategy for comprehensive glycoproteome analysis using hydrazide chemistry based method. *Sci. Rep.* **5**, 1–15
339. Wang, X., Xia, N., and Liu, L. (2013) Boronic acid-based approach for separation and immobilization of glycoproteins and its application in sensing. *Int. J. Mol. Sci.* **14**, 20890–20912
340. Espina-Benitez, M. B., Randon, J., Demesmay, C., and Dugas, V. (2018) Back to BAC: insights into boronate affinity chromatography interaction mechanisms. *Sep. Purif. Rev.* **47**, 214–228
341. Bull, S. D., Davidson, M. G., Van Den Elsen, J. M. H., Fossey, J. S., Jenkins, A. T. A., Jiang, Y. B., Kubo, Y., Marken, F., Sakurai, K., Zhao, J., and James, T. D. (2013) Exploiting the reversible covalent bonding of boronic acids: recognition, sensing, and assembly. *Acc. Chem. Res.* **46**, 312–326
342. Xu, Y., Zhang, L., and Lu, H. (2013) Use of boronic acid nanoparticles in glycoprotein enrichment. *Methods Mol. Biol.* **951**, 45–55
343. Zhang, L., Xu, Y., Yao, H., Xie, L., Yao, J., Lu, H., and Yang, P. (2009) Boronic acid functionalized core-satellite composite nanoparticles for advanced enrichment of glycopeptides and glycoproteins. *Chemistry* **15**, 10158–10166
344. Xu, Y., Wu, Z., Zhang, L., Lu, H., Yang, P., Webley, P. A., and Zhao, D. (2009) Highly specific enrichment of glycopeptides using boronic acid-functionalized mesoporous silica. *Anal. Chem.* **81**, 503–508
345. Chen, W., Smeekens, J. M., and Wu, R. (2014) A universal chemical enrichment method for mapping the yeast N-glycoproteome by mass spectrometry (MS). *Mol. Cell. Proteomics* **13**, 1563–1572
346. Zhang, Y., Jing, H., Wen, T., Wang, Y., Zhao, Y., Wang, X., Qian, X., and Ying, W. (2019) Phenylboronic acid functionalized C3N4 facultative hydrophilic materials for enhanced enrichment of glycopeptides. *Talanta* **191**, 509–518
347. Xiao, H., Tang, G. X., Chen, W., and Wu, R. (2015). In: *Neuromethods*, Humana Press Inc, New York, NY: 31–41
348. Xiao, H., Hwang, J. E., and Wu, R. (2018) Mass spectrometric analysis of the N-glycoproteome in statin-treated liver cells with two lectin-independent chemical enrichment methods. *Int. J. Mass Spectrom.* **429**, 66–75
349. Guy, C. S., Gibson, M. I., and Fullam, E. (2019) Targeting extracellular glycans: tuning multimeric boronic acids for pathogen-selective killing of Mycobacterium tuberculosis. *Chem. Sci.* **10**, 5935–5942
350. Wang, H., Bie, Z., Lü, C., and Liu, Z. (2013) Magnetic nanoparticles with dendrimer-assisted boronate avidity for the selective enrichment of trace glycoproteins. *Chem. Sci.* **4**, 4298–4303
351. Xiao, H., Chen, W., Smeekens, J. M., and Wu, R. (2018) An enrichment method based on synergistic and reversible covalent interactions for large-scale analysis of glycoproteins. *Nat. Commun.* **9**, 1692
352. Malaker, S. A., Penny, S. A., Steadman, L. G., Myers, P. T., Loke, J. C., Raghavan, M., Bai, D. L., Shabanowitz, J., Hunt, D. F., and Cobbold, M. (2017) Identification of glycopeptides as posttranslationally modified neoantigens in Leukemia. *Cancer Immunol. Res.* **5**, 376–384
353. Xiong, Y., Li, X., Li, M., Qin, H., Chen, C., Wang, D., Wang, X., Zheng, X., Liu, Y., Liang, X., and Qing, G. (2020) What is hidden behind Schiff base hydrolysis? Dynamic covalent chemistry for precise capture of sialylated glycans. *J. Am. Chem. Soc.* **142**, 7627–7637
354. Lee, H. H. L., Heo, C. E., Seo, N., Yun, S. G., An, H. J., and Kim, H. I. (2018) Accurate quantification of *N* -glycolylneuraminic acid in therapeutic proteins using supramolecular mass spectrometry. *J. Am. Chem. Soc.* **140**, 16528–16534
355. Palaniappan, K. K., and Bertozzi, C. R. (2016) Chemical glycoproteomics. *Chem. Rev.* **116**, 14277–14306
356. Chuh, K. N., and Pratt, M. R. (2015) Chemical methods for the proteome-wide identification of posttranslationally modified proteins. *Curr. Opin. Chem. Biol.* **24**, 27–37
357. Sletten, E. M., and Bertozzi, C. R. (2011) From mechanism to mouse: a tale of two bioorthogonal reactions. *Acc. Chem. Res.* **44**, 666–676
358. Huang, T., Armbruster, M. R., Coulton, J. B., and Edwards, J. L. (2019) Chemical tagging in mass spectrometry for systems biology. *Anal. Chem.* **91**, 109–125
359. Sletten, E. M., and Bertozzi, C. R. (2009) Bioorthogonal chemistry: fishing for selectivity in a sea of functionality. *Angew. Chem. Int. Ed. Engl.* **48**, 6974–6998
360. Debets, M. F., Van Hest, J. C. M., and Rutjes, F. P. J. T. (2013) Bio-orthogonal labelling of biomolecules: new functional handles and ligation methods. *Org. Biomol. Chem.* **11**, 6439–6455
361. Parker, C. G., and Pratt, M. R. (2020) Click chemistry in proteomic investigations. *Cell* **180**, 605–632
362. Saxon, E., and Bertozzi, C. R. (2000) Cell surface engineering by a modified Staudinger reaction. *Science* **287**, 2007–2010
363. Rostovtsev, V. V., Green, L. G., Fokin, V. V., and Sharpless, K. B. (2002) A stepwise Huisgen cycloaddition process: copper(I)-catalyzed

regioselective “ligation” of azides and terminal alkynes. *Angew. Chem. Int. Ed. Engl.* **41**, 2596–2599
364. Agard, N. J., Prescher, J. A., and Bertozzi, C. R. (2004) A strain-promoted [3 + 2] azide-alkyne cycloaddition for covalent modification of biomolecules in living systems. *J. Am. Chem. Soc.* **126**, 15046–15047
365. Tornøe, C. W., Christensen, C., and Meldal, M. (2002) Peptidotriazoles on solid phase: [1,2,3]-triazoles by regiospecific copper(I)-catalyzed 1,3-dipolar cycloadditions of terminal alkynes to azides. *J. Org. Chem.* **67**, 3057–3064
366. Agard, N. J., Baskin, J. M., Prescher, J. A., Lo, A., and Bertozzi, C. R. (2006) A comparative study of bioorthogonal reactions with azides. *ACS Chem. Biol.* **1**, 644–648
367. Prescher, J. A., Dube, D. H., and Bertozzi, C. R. (2004) Chemical remodelling of cell surfaces in living animals. *Nature* **430**, 873–877
368. Hsu, T. L., Hanson, S. R., Kishikawa, K., Wang, S. K., Sawa, M., and Wong, C. H. (2007) Alkynyl sugar analogs for the labeling and visualization of glycoconjugates in cells. *Proc. Natl. Acad. Sci. U. S. A.* **104**, 2614–2619
369. Han, S., Collins, B. E., Bengtson, P., and Paulson, J. C. (2005) Homomultimeric complexes of CD22 in B cells revealed by protein-glycan cross-linking. *Nat. Chem. Biol.* **1**, 93–97
370. Hang, H. C., Yu, C., Kato, D. L., and Bertozzi, C. R. (2003) A metabolic labeling approach toward proteomic analysis of mucin-type O-linked glycosylation. *Proc. Natl. Acad. Sci. U. S. A.* **100**, 14846–14851
371. Boyce, M., Carrico, I. S., Ganguli, A. S., Yu, S. H., Hangauer, M. J., Hubbard, S. C., Kohler, J. J., and Bertozzi, C. R. (2011) Metabolic cross-talk allows labeling of O-linked β-N- acetylglucosamine-modified proteins via the N-acetylgalactosamine salvage pathway. *Proc. Natl. Acad. Sci. U. S. A.* **108**, 3141–3146
372. Luchansky, S. J., Goon, S., and Bertozzi, C. R. (2004) Expanding the diversity of unnatural cell-surface sialic acids. *ChemBioChem* **5**, 371–374
373. Dube, D. H., Prescher, J. A., Quang, C. M., and Bertozzi, C. R. (2006) Probing mucin-type O-linked glycosylation in living animals. *Proc. Natl. Acad. Sci. U. S. A.* **103**, 4819–4824
374. Rabuka, D., Hubbard, S. C., Laughlin, S. T., Argade, S. P., and Bertozzi, C. R. (2006) A chemical reporter strategy to probe glycoprotein fucosylation. *J. Am. Chem. Soc.* **128**, 12078–12079
375. Sawa, M., Hsu, T. L., Itoh, T., Sugiyama, M., Hanson, S. R., Vogt, P. K., and Wong, C. H. (2006) Glycoproteomic probes for fluorescent imaging of fucosylated glycans *in vivo*. *Proc. Natl. Acad. Sci. U. S. A.* **103**, 12371–12376
376. Gurcel, C., Vercoutter-Edouart, A. S., Fonbonne, C., Mortuaire, M., Salvador, A., Michalski, J. C., and Lemoine, J. (2008) Identification of new O-GlcNAc modified proteins using a click-chemistry-based tagging. *Anal. Bioanal. Chem.* **390**, 2089–2097
377. Zaro, B. W., Yang, Y. Y., Hang, H. C., and Pratt, M. R. (2011) Chemical reporters for fluorescent detection and identification of O-GlcNAc-modified proteins reveal glycosylation of the ubiquitin ligase NEDD4-1. *Proc. Natl. Acad. Sci. U. S. A.* **108**, 8146–8151
378. Vocadlo, D. J., Hang, H. C., Kim, E. J., Hanover, J. A., and Bertozzi, C. R. (2003) A chemical approach for identifying O-GlcNAc-modified proteins in cells. *Proc. Natl. Acad. Sci. U. S. A.* **100**, 9116–9121
379. Zaro, B. W., Chuh, K. N., and Pratt, M. R. (2014) Chemical reporter for visualizing metabolic cross-talk between carbohydrate metabolism and protein modification. *ACS Chem. Biol.* **9**, 1991–1996
380. Pedowitz, N. J., Zaro, B. W., and Pratt, M. R. (2020) Metabolic labeling for the visualization and identification of potentially O-GlcNAc-modified proteins. *Curr. Protoc. Chem. Biol.* **12**, e81
381. Darabedian, N., Gao, J., Chuh, K. N., Woo, C. M., and Pratt, M. R. (2018) The metabolic chemical reporter 6-azido-6-deoxy-glucose further reveals the substrate promiscuity of O-GlcNAc transferase and catalyzes the discovery of intracellular protein modification by O-glucose. *J. Am. Chem. Soc.* **140**, 7092–7100
382. Chuh, K. N., Zaro, B. W., Piller, F., Piller, V., and Pratt, M. R. (2014) Changes in metabolic chemical reporter structure yield a selective probe of O -GlcNAc modification. *J. Am. Chem. Soc.* **136**, 12283–12295
383. Darabedian, N., Yang, B., Ding, R., Cutolo, G., Zaro, B. W., Woo, C. M., and Pratt, M. R. (2020) O-acetylated chemical reporters of glycosylation can display metabolism-dependent background labeling of proteins but are generally reliable tools for the identification of glycoproteins. *Front. Chem.* **8**, 318
384. Xiao, H., Tang, G. X., and Wu, R. (2016) Site-specific quantification of surface N-glycoproteins in statin-treated liver cells. *Anal. Chem.* **88**, 3324–3332
385. Li, H., Li, L., Cheng, K., Ning, Z., Mayne, J., Zhang, X., Walker, K., Chen, R., Twine, S., Li, J., and Figeys, D. (2020) Chemoenzymatic method for glycoproteomic N-glycan type quantitation. *Anal. Chem.* **92**, 1618–1627
386. Slade, P. G., Hajivandi, M., Bartel, C. M., and Gorfien, S. F. (2012) Identifying the CHO secretome using mucin-type O-linked glycosylation and click-chemistry. *J. Proteome Res.* **11**, 6175–6186
387. Woo, C. M., Lund, P. J., Huang, A. C., Davis, M. M., Bertozzi, C. R., and Pitteri, S. J. (2018) Mapping and quantification of over 2000 O-linked glycopeptides in activated human T cells with isotope-targeted glycoproteomics (ISOTAG). *Mol. Cell. Proteomics* **17**, 764–775
388. Schumann, B., Malaker, S. A., Wisnovsky, S. P., Debets, M. F., Agbay, A. J., Fernandez, D., Wagner, L. J. S., Lin, L., Li, Z., Choi, J., Fox, D. M., Peh, J., Gray, M. A., Pedram, K., Kohler, J. J., *et al*. (2020) Bump-and-Hole engineering identifies specific substrates of glycosyltransferases in living cells. *Mol. Cell* **78**, 824–834.e15
389. Woo, C. M., Iavarone, A. T., Spiciarich, D. R., Palaniappan, K. K., and Bertozzi, C. R. (2015) Isotope-targeted glycoproteomics (IsoTaG): a mass-independent platform for intact N- and O-glycopeptide discovery and analysis. *Nat. Methods* **12**, 561–567
390. Woo, C. M., Felix, A., Zhang, L., Elias, J. E., and Bertozzi, C. R. (2017) Isotope-targeted glycoproteomics (IsoTaG) analysis of sialylated N- and O-glycopeptides on an orbitrap fusion tribrid using azido and alkynyl sugars. *Anal. Bioanal. Chem.* **409**, 579–588
391. Woo, C. M., Felix, A., Byrd, W. E., Zuegel, D. K., Ishihara, M., Azadi, P., Iavarone, A. T., Pitteri, S. J., and Bertozzi, C. R. (2017) Development of IsoTaG, a chemical glycoproteomics technique for profiling intact N- and O-glycopeptides from whole cell proteomes. *J. Proteome Res.* **16**, 1706–1718
392. Hubbard, S. C., Boyce, M., McVaugh, C. T., Peehl, D. M., and Bertozzi, C. R. (2011) Cell surface glycoproteomic analysis of prostate cancer-derived PC-3 cells. *Bioorg. Med. Chem. Lett.* **21**, 4945–4950
393. Chen, W., Smeekens, J. M., and Wu, R. (2015) Systematic and site-specific analysis of N-sialoglycosylated proteins on the cell surface by integrating click chemistry and MS-based proteomics. *Chem. Sci.* **6**, 4681–4689
394. Smeekens, J. M., Chen, W., and Wu, R. (2015) Mass spectrometric analysis of the cell surface N-glycoproteome by combining metabolic labeling and click chemistry. *J. Am. Soc. Mass Spectrom.* **26**, 604–614
395. Hahne, H., Sobotzki, N., Nyberg, T., Helm, D., Borodkin, V. S., Van Aalten, D. M. F., Agnew, B., and Kuster, B. (2013) Proteome wide purification and identification of O-GlcNAc-modified proteins using click chemistry and mass spectrometry. *J. Proteome Res.* **12**, 927–936
396. Nandi, A., Sprung, R., Barma, D. K., Zhao, Y., Kim, S. C., Falck, J. R., and Zhao, Y. (2006) Global identification of O-GlcNAc-modified proteins. *Anal. Chem.* **78**, 452–458
397. Ramirez, D. H., Aonbangkhen, C., Wu, H. Y., Naftaly, J. A., Tang, S., O'Meara, T. R., and Woo, C. M. (2020) Engineering a proximity-directed O-GlcNAc transferase for selective protein O-GlcNAcylation in cells. *ACS Chem. Biol.* **15**, 1059–1066
398. Spiciarich, D. R., Nolley, R., Maund, S. L., Purcell, S. C., Herschel, J., Iavarone, A. T., Peehl, D. M., and Bertozzi, C. R. (2017) Bioorthogonal labeling of human prostate cancer tissue slice cultures for glycoproteomics. *Angew. Chem. Int. Ed. Engl.* **56**, 8992–8997
399. Lopez Aguilar, A., Briard, J. G., Yang, L., Ovryn, B., Macauley, M. S., and Wu, P. (2017) Tools for studying glycans: recent advances in chemoenzymatic glycan labeling. *ACS Chem. Biol.* **12**, 611–621
400. Khidekel, N., Arndt, S., Lamarre-Vincent, N., Lippert, A., Poulin-Kerstien, K. G., Ramakrishnan, B., Qasba, P. K., and Hsieh-Wilson, L. C. (2003) A chemoenzymatic approach toward the rapid and sensitive detection of O-GlcNAc posttranslational modifications. *J. Am. Chem. Soc.* **125**, 16162–16163
401. Tai, H. C., Khidekel, N., Ficarro, S. B., Peters, E. C., and Hsieh-Wilson, L. C. (2004) Parallel identification of O-GlcNAc-modified proteins from cell lysates. *J. Am. Chem. Soc.* **126**, 10500–10501
402. Wen, L., Liu, D., Zheng, Y., Huang, K., Cao, X., Song, J., and Wang, P. G. (2018) A one-step chemoenzymatic labeling strategy for probing sialylated thomsen-friedenreich antigen. *ACS Cent. Sci.* **4**, 451–457

403. Ma, J., Wang, W. H., Li, Z., Shabanowitz, J., Hunt, D. F., and Hart, G. W. (2019) O-GlcNAc site mapping by using a combination of chemoenzymatic labeling, copper-free click chemistry, reductive cleavage, and electron-transfer dissociation mass spectrometry. *Anal. Chem.* **91**, 2620–2625
404. Wang, Z., Udeshi, N. D., Slawson, C., Compton, P. D., Sakabe, K., Cheung, W. D., Shabanowitz, J., Hunt, D. F., and Hart, G. W. (2010) Extensive crosstalk between O-GlcNAcylation and phosphorylation regulates cytokinesis. *Sci. Signal.* **3**, ra2
405. Griffin, M. E., Jensen, E. H., Mason, D. E., Jenkins, C. L., Stone, S. E., Peters, E. C., and Hsieh-Wilson, L. C. (2016) Comprehensive mapping of O-GlcNAc modification sites using a chemically cleavable tag. *Mol. Biosyst.* **12**, 1756–1759
406. Thompson, J. W., Griffin, M. E., and Hsieh-Wilson, L. C. (2018). In: *Methods in Enzymology*, Academic Press Inc, Cambridge, MA: 101–135
407. Li, J., Li, Z., Duan, X., Qin, K., Dang, L., Sun, S., Cai, L., Hsieh-Wilson, L. C., Wu, L., and Yi, W. (2019) An isotope-coded photocleavable probe for quantitative profiling of protein O-GlcNAcylation. *ACS Chem. Biol.* **14**, 4–10
408. Wang, Z., Udeshi, N. D., O'Malley, M., Shabanowitz, J., Hunt, D. F., and Hart, G. W. (2010) Enrichment and site mapping of O-linked N-acetylglucosamine by a combination of chemical/enzymatic tagging, photochemical cleavage, and electron transfer dissociation mass spectrometry. *Mol. Cell. Proteomics* **9**, 153–160
409. Dehennaut, V., Slomianny, M. C., Page, A., Vercoutter-Edouart, A. S., Jessus, C., Michalski, J. C., Vilain, J. P., Bodart, J. F., and Lefebvre, T. (2008) Identification of structural and functional O-linked N-acetylglucosamine-bearing proteins in Xenopus laevis oocyte. *Mol. Cell. Proteomics* **7**, 2229–2245
410. Khidekel, N., Ficarro, S. B., Clark, P. M., Bryan, M. C., Swaney, D. L., Rexach, J. E., Sun, Y. E., Coon, J. J., Peters, E. C., and Hsieh-Wilson, L. C. (2007) Probing the dynamics of O-GlcNAc glycosylation in the brain using quantitative proteomics. *Nat. Chem. Biol.* **3**, 339–348
411. Parker, B. L., Gupta, P., Cordwell, S. J., Larsen, M. R., and Palmisano, G. (2011) Purification and identification of O-GlcNAc-modified peptides using phosphate-based alkyne CLICK chemistry in combination with titanium dioxide chromatography and mass spectrometry. *J. Proteome Res.* **10**, 1449–1458
412. Xu, S., Sun, F., and Wu, R. (2020) A chemoenzymatic method based on easily accessible enzymes for profiling protein O-GlcNAcylation. *Anal. Chem.* **92**, 9807–9814
413. Lopez Aguilar, A., Meng, L., Hou, X., Li, W., Moremen, K. W., and Wu, P. (2018) Sialyltransferase-based chemoenzymatic histology for the detection of N - and O -glycans. *Bioconjug. Chem.* **29**, 1231–1239
414. Hong, S., Shi, Y., Wu, N. C., Grande, G., Douthit, L., Wang, H., Zhou, W., Sharpless, K. B., Wilson, I. A., Xie, J., and Wu, P. (2019) Bacterial glycosyltransferase-mediated cell-surface chemoenzymatic glycan modification. *Nat. Commun.* **10**, 1–11
415. Lantos, A. B., Carlevaro, G., Araoz, B., Ruiz Diaz, P., Camara, M. d l M., Buscaglia, C. A., Bossi, M., Yu, H., Chen, X., Bertozzi, C. R., Mucci, J., and Campetella, O. (2016) Sialic acid glycobiology unveils Trypanosoma cruzi trypomastigote membrane physiology. *PLOS Pathog.* **12**, e1005559
416. Muiá, R. P., Yu, H., Prescher, J. A., Hellman, U., Chen, X., Bertozzi, C. R., and Campetella, O. (2010) Identification of glycoproteins targeted by Trypanosoma cruzi trans-sialidase, a virulence factor that disturbs lymphocyte glycosylation. *Glycobiology* **20**, 833–842
417. Yang, W., Ao, M., Song, A., Xu, Y., Sokoll, L., and Zhang, H. (2020) Mass spectrometric mapping of glycoproteins modified by Tn-antigen using solid-phase capture and enzymatic release. *Anal. Chem.* **92**, 9230–9238
418. Mbua, N. E., Li, X., Flanagan-Steet, H. R., Meng, L., Aoki, K., Moremen, K. W., Wolfert, M. A., Steet, R., and Boons, G.-J. (2013) Selective exo-enzymatic labeling of N-glycans on the surface of living cells by recombinant ST6Gal I. *Angew. Chem. Int. Ed. Engl.* **52**, 13012–13015
419. Yu, S. H., Zhao, P., Sun, T., Gao, Z., Moremen, K. W., Boons, G. J., Wells, L., and Steet, R. (2016) Selective exo-enzymatic labeling detects increased cell surface sialoglycoprotein expression upon megakaryocytic differentiation. *J. Biol. Chem.* **291**, 3982–3989
420. Zhu, H., Wang, S., Liu, D., Ding, L., Chen, C., Liu, Y., Wu, Z., Bollag, R., Liu, K., Alexander, W. M., Yin, J., Ma, C., Li, L., and Wang, P. G. (2020) Identifying sialylation linkages at the glycopeptide level by glycosyltransferase labeling assisted mass spectrometry (GLAMS). *Anal. Chem.* **92**, 6297–6303
421. Devaraj, N. K. (2018) The future of bioorthogonal chemistry. *ACS Cent. Sci.* **4**, 952–959
422. Mancera-Arteu, M., Giménez, E., Benavente, F., Barbosa, J., and Sanz-Nebot, V. (2017) Analysis of O-glycopeptides by acetone enrichment and capillary electrophoresis-mass spectrometry. *J. Proteome Res.* **16**, 4166–4176
423. Wang, G., De Jong, R. N., Van Den Bremer, E. T. J., Parren, P. W. H. I., and Heck, A. J. R. (2017) Enhancing accuracy in molecular weight determination of highly heterogeneously glycosylated proteins by native tandem mass spectrometry. *Anal. Chem.* **89**, 4793–4797
424. Yang, Y., Wang, G., Song, T., Lebrilla, C. B., and Heck, A. J. R. (2017) Resolving the micro-heterogeneity and structural integrity of monoclonal antibodies by hybrid mass spectrometric approaches. *MAbs* **9**, 638–645
425. Lin, Y. H., Franc, V., and Heck, A. J. R. (2018) Similar albeit not the same: in-depth analysis of proteoforms of human serum, bovine serum, and recombinant human fetuin. *J. Proteome Res.* **17**, 2861–2869
426. Franc, V., Yang, Y., and Heck, A. J. R. (2017) Proteoform profile mapping of the human serum complement component C9 revealing unexpected new features of N-, O-, and C-glycosylation. *Anal. Chem.* **89**, 3483–3491
427. Yang, Y., Liu, F., Franc, V., Halim, L. A., Schellekens, H., and Heck, A. J. R. (2016) Hybrid mass spectrometry approaches in glycoprotein analysis and their usage in scoring biosimilarity. *Nat. Commun.* **7**, 1–10
428. Bern, M., Caval, T., Kil, Y. J., Tang, W., Becker, C., Carlson, E., Kletter, D., Sen, K. I., Galy, N., Hagemans, D., Franc, V., and Heck, A. J. R. (2018) Parsimonious charge deconvolution for native mass spectrometry. *J. Proteome Res.* **17**, 1216–1226
429. Lin, Y. H., Zhu, J., Meijer, S., Franc, V., and Heck, A. J. R. (2019) Glycoproteogenomics: a frequent gene polymorphism affects the glycosylation pattern of the human serum fetuin/α-2-HS-glycoprotein. *Mol. Cell. Proteomics* **18**, 1479–1490
430. Leney, A. C., Rafie, K., Van Aalten, D. M. F., and Heck, A. J. R. (2017) Direct monitoring of protein O-GlcNAcylation by high-resolution native mass spectrometry. *ACS Chem. Biol.* **12**, 2078–2084
431. Čaval, T., Tian, W., Yang, Z., Clausen, H., and Heck, A. J. R. (2018) Direct quality control of glycoengineered erythropoietin variants. *Nat. Commun.* **9**, 1–8
432. Wörner, T. P., Snijder, J., Bennett, A., Agbandje-McKenna, M., Makarov, A. A., and Heck, A. J. R. (2020) Resolving heterogeneous macromolecular assemblies by orbitrap-based single-particle charge detection mass spectrometry. *Nat. Methods* **17**, 395–398
433. Chen, Z., Glover, M. S., and Li, L. (2018) Recent advances in ion mobility–mass spectrometry for improved structural characterization of glycans and glycoconjugates. *Curr. Opin. Chem. Biol.* **42**, 1–8
434. Gelb, A. S., Lai, R., Li, H., and Dodds, E. D. (2019) Composition and charge state influence on the ion-neutral collision cross sections of protonated N-linked glycopeptides: an experimental and theoretical deconstruction of coulombic repulsion: vs. charge solvation effects. *Analyst* **144**, 5738–5747
435. Mookherjee, A., and Guttman, M. (2018) Bridging the structural gap of glycoproteomics with ion mobility spectrometry. *Curr. Opin. Chem. Biol.* **42**, 86–92
436. Harvey, D. J., Scarff, C. A., Edgeworth, M., Crispin, M., Scanlan, C. N., Sobott, F., Allman, S., Baruah, K., Pritchard, L., and Scrivens, J. H. (2013) Travelling wave ion mobility and negative ion fragmentation for the structural determination of *N* -linked glycans. *Electrophoresis* **34**, 2368–2378
437. Glaskin, R. S., Khatri, K., Wang, Q., Zaia, J., and Costello, C. E. (2017) Construction of a database of collision cross section values for glycopeptides, glycans, and peptides determined by IM-MS. *Anal. Chem.* **89**, 4452–4460
438. Kolli, V., Schumacher, K. N., and Dodds, E. D. (2017) Ion mobility-resolved collision-induced dissociation and electron transfer dissociation of: N-glycopeptides: gathering orthogonal connectivity information from a single mass-selected precursor ion population. *Analyst* **142**, 4691–4702
439. Barroso, A., Giménez, E., Konijnenberg, A., Sancho, J., Sanz-Nebot, V., and Sobott, F. (2018) Evaluation of ion mobility for the separation of glycoconjugate isomers due to different types of sialic acid linkage, at

the intact glycoprotein, glycopeptide and glycan level. *J. Proteomics* **173**, 22–31

440. Zhu, F., Trinidad, J. C., and Clemmer, D. E. (2015) Glycopeptide site heterogeneity and structural diversity determined by combined lectin affinity chromatography/IMS/CID/MS techniques. *J. Am. Soc. Mass Spectrom.* **26**, 1092–1102
441. Campbell, J. L., Baba, T., Liu, C., Lane, C. S., Yves, J. C., Blanc, L., and Hager, J. W. (2017) Analyzing glycopeptide isomers by combining differential mobility spectrometry with electron-and collision-based tandem mass spectrometry. *J. Am. Soc. Mass Spectrom.* **28**, 1374–1381
442. Sarbu, M., Zhu, F., Peter-Katalinić, J., Clemmer, D. E., and Zamfir, A. D. (2015) Application of ion mobility tandem mass spectrometry to compositional and structural analysis of glycopeptides extracted from the urine of a patient diagnosed with Schindler disease. *Rapid Commun. Mass Spectrom.* **29**, 1929–1937
443. Both, P., Green, A. P., Gray, C. J., Šardzík, R., Voglmeir, J., Fontana, C., Austeri, M., Rejzek, M., Richardson, D., Field, R. A., Widmalm, G., Flitsch, S. L., and Eyers, C. E. (2014) Discrimination of epimeric glycans and glycopeptides using IM-MS and its potential for carbohydrate sequencing. *Nat. Chem.* **6**, 65–74
444. Creese, A. J., and Cooper, H. J. (2012) Separation and identification of isomeric glycopeptides by high field asymmetric waveform ion mobility spectrometry. *Anal. Chem.* **84**, 2597–2601
445. Pathak, P., Baird, M. A., and Shvartsburg, A. A. (2020) High-resolution ion mobility separations of isomeric glycoforms with variations on the peptide and glycan levels. *J. Am. Soc. Mass Spectrom.* **31**, 1603–1609
446. Yen, H., Liko, I., Gault, J., Wu, D., Struwe, W. B., and Robinson, C. V. (2020) Correlating glycoforms of DC-SIGN with stability using a combination of enzymatic digestion and ion mobility mass spectrometry. *Angew. Chem. Int. Ed. Engl.* https://doi.org/10.1002/ange.202005727
447. Guttman, M., and Lee, K. K. (2016) Site-specific mapping of sialic acid linkage isomers by ion mobility spectrometry. *Anal. Chem.* **88**, 5212–5217
448. Hinneburg, H., Hofmann, J., Struwe, W. B., Thader, A., Altmann, F., Varón Silva, D., Seeberger, P. H., Pagel, K., and Kolarich, D. (2016) Distinguishing N-acetylneuraminic acid linkage isomers on glycopeptides by ion mobility-mass spectrometry. *Chem. Commun.* **52**, 4381–4384
449. Hebert, A. S., Prasad, S., Belford, M. W., Bailey, D. J., McAlister, G. C., Abbatiello, S. E., Huguet, R., Wouters, E. R., Dunyach, J. J., Brademan, D. R., Westphall, M. S., and Coon, J. J. (2018) Comprehensive single-shot proteomics with FAIMS on a hybrid orbitrap mass spectrometer. *Anal. Chem.* **90**, 9529–9537
450. Waas, M., Bhattacharya, S., Chuppa, S., Wu, X., Jensen, D. R., Omasits, U., Wollscheid, B., Volkman, B. F., Noon, K. R., and Gundry, R. L. (2014) Combine and conquer: surfactants, solvents, and chaotropes for robust mass spectrometry based analyses of membrane proteins. *Anal. Chem.* **86**, 1551–1559
451. Waas, M., Pereckas, M., Jones Lipinski, R. A., Ashwood, C., and Gundry, R. L. (2019) SP2: rapid and automatable contaminant removal from peptide samples for proteomic analyses. *J. Proteome Res.* **18**, 1644–1656
452. Brown, K. A., Chen, B., Guardado-Alvarez, T. M., Lin, Z., Hwang, L., Ayaz-Guner, S., Jin, S., and Ge, Y. (2019) A photocleavable surfactant for top-down proteomics. *Nat. Methods* **16**, 417–420
453. Brown, K. A., Tucholski, T., Eken, C., Knott, S., Zhu, Y., Jin, S., and Ge, Y. (2020) High-throughput proteomics enabled by a photocleavable surfactant. *Angew. Chem. Int. Ed. Engl.* **132**, 8484–8488
454. Zougman, A., Wilson, J. P., Roberts, L. D., and Banks, R. E. (2020) Detergent-free simultaneous sample preparation method for proteomics and metabolomics. *J. Proteome Res.* **19**, 2838–2844
455. Barallobre-Barreiro, J., Baig, F., Fava, M., Yin, X., and Mayr, M. (2017) Glycoproteomics of the extracellular matrix: a method for intact glycopeptide analysis using mass spectrometry. *J. Vis. Exp.*, 55674
456. Hailemariam, M., Eguez, R. V., Singh, H., Bekele, S., Ameni, G., Pieper, R., and Yu, Y. (2018) S-trap, an ultrafast sample-preparation approach for shotgun proteomics. *J. Proteome Res.* **17**, 2917–2924
457. Ludwig, K. R., Schroll, M. M., and Hummon, A. B. (2018) Comparison of in-solution, FASP, and S-trap based digestion methods for bottom-up proteomic studies. *J. Proteome Res.* **17**, 2480–2490
458. Wiśniewski, J. R., Zougman, A., Nagaraj, N., and Mann, M. (2009) Universal sample preparation method for proteome analysis. *Nat. Methods* **6**, 359–362
459. Chen, S. Y., Dong, M., Yang, G., Zhou, Y., Clark, D. J., Lih, T. M., Schnaubelt, M., Liu, Z., and Zhang, H. (2020) Glycans, glycosite, and intact glycopeptide analysis of N-linked glycoproteins using liquid handling systems. *Anal. Chem.* **92**, 1680–1686
460. Sethi, M. K., Downs, M., and Zaia, J. (2020) Serial in-solution digestion protocol for mass spectrometry-based glycomics and proteomics analysis. *Mol. Omics* **16**, 364–376
461. Ramesh, B., Abnouf, S., Mali, S., Moree, W. J., Patil, U., Bark, S. J., and Varadarajan, N. (2019) Engineered ChymotrypsiN for mass spectrometry-based detection of protein glycosylation. *ACS Chem. Biol.* **14**, 2616–2628
462. Kolarich, D., Jensen, P. H., Altmann, F., and Packer, N. H. (2012) Determination of site-specific glycan heterogeneity on glycoproteins. *Nat. Protoc.* **7**, 1285–1298
463. Pralow, A., Hoffmann, M., Nguyen-Khuong, T., Rapp, E., and Reichl, U. (2017) Improvement of the glycoproteomic toolbox with the discovery of a unique C-terminal cleavage specificity of flavastacin for N-glycosylated asparagines. *Sci. Rep.* **7**, 1–9
464. Sun, Z., Sun, D., Wang, F., Cheng, K., Zhang, Z., Xu, B., Ye, M., Wang, L., and Zou, H. (2014) Differential analysis of N-glycoproteome between hepatocellular carcinoma and normal human liver tissues by combination of multiple protease digestion and solid phase based labeling. *Clin. Proteomics* **11**, 26
465. Stavenhagen, K., Kayili, H. M., Holst, S., Koeleman, C. A. M., Engel, R., Wouters, D., Zeerleder, S., Salih, B., and Wuhrer, M. (2018) N- and O-glycosylation analysis of human C1-inhibitor reveals extensive mucin-type O-glycosylation. *Mol. Cell. Proteomics* **17**, 1225–1238
466. Malaker, S. A., Pedram, K., Ferracane, M. J., Bensing, B. A., Krishnan, V., Pett, C., Yu, J., Woods, E. C., Kramer, J. R., Westerlind, U., Dorigo, O., and Bertozzi, C. R. (2019) The mucin-selective protease StcE enables molecular and functional analysis of human cancer-associated mucins. *Proc. Natl. Acad. Sci. U. S. A.* **116**, 7278–7287
467. Shon, D. J., Malaker, S., Pedram, K., Yang, E., Krishnan, V., Dorigo, O., and Bertozzi, C. (2020) An Enzymatic Toolkit for Selective Proteolysis, Detection, and Visualization of Mucin-Domain Glycoproteins. *PNAS* **117**, 21299–21307
468. Haurat, M. F., Scott, N. E., Venanzio, G., Di, Lopez, J., Pluvinage, B., Boraston, A. B., Ferracane, M. J., and Feldman, M. F. (2020) *The Glycoprotease CpaA Secreted by Medically Relevant Acinetobacter Species Targets Multiple O-Linked Host Glycoproteins*. https://doi.org/10.1128/mBio.02033-20
469. Malaker, S. A., Shon, J., Pedram, K., Riley, N. M., and Bertozzi, C. R. (2019). *Molecular & Cellular Proteomics Conference,* S42. https://doi.org/10.1101/2021.01.27.428510
470. Hinneburg, H., Stavenhagen, K., Schweiger-Hufnagel, U., Pengelley, S., Jabs, W., Seeberger, P. H., Silva, D. V., Wuhrer, M., and Kolarich, D. (2016) The art of destruction: optimizing collision energies in quadrupole-time of flight (Q-TOF) instruments for glycopeptide-based glycoproteomics. *J. Am. Soc. Mass Spectrom.* **27**, 507–519
471. Hoffmann, M., Pioch, M., Pralow, A., Hennig, R., Kottler, R., Reichl, U., and Rapp, E. (2018) The fine art of destruction: a guide to in-depth glycoproteomic analyses—exploiting the diagnostic potential of fragment ions. *Proteomics* **18**, 1800282
472. Wang, Y., and Tian, Z. (2020) New energy setup strategy for intact N-glycopeptides characterization using higher-energy collisional dissociation. *J. Am. Soc. Mass Spectrom.* **31**, 651–657
473. Riley, N., Malaker, S. A., Driessen, M. D., and Bertozzi, C. (2020) Optimal Dissociation Methods Differ for N- and O-Glycopeptides. *J. Proteome Res.* **19**, 3286–3301
474. Syka, J. E. P., Coon, J. J., Schroeder, M. J., Shabanowitz, J., and Hunt, D. F. (2004) Peptide and protein sequence analysis by electron transfer dissociation mass spectrometry. *Proc. Natl. Acad. Sci. U. S. A.* **101**, 9528–9533
475. Zubarev, R., Kelleher, N. L., and McLafferty, F. W. (1998) Electron capture dissociation of multiply charged protein cations. A nonergodic process. *J. Am. Chem. Soc.* **120**, 3265–3266
476. Zhao, P., Viner, R., Teo, C. F., Boons, G. J., Horn, D., and Wells, L. (2011) Combining high-energy C-trap dissociation and electron transfer

dissociation for protein O-GlcNAc modification site assignment. *J. Proteome Res.* **10**, 4088–4104
477. Wu, S. W., Pu, T. H., Viner, R., and Khoo, K. H. (2014) Novel LC-MS2 product dependent parallel data acquisition function and data analysis workflow for sequencing and identification of intact glycopeptides. *Anal. Chem.* **86**, 5478–5486
478. Saba, J., Dutta, S., Hemenway, E., and Viner, R. (2012) Increasing the productivity of glycopeptides analysis by using higher-energy collision dissociation-accurate mass-product-dependent electron transfer dissociation. *Int. J. Proteomics* **2012**, 560391
479. Singh, C., Zampronio, C. G., Creese, A. J., and Cooper, H. J. (2012) Higher energy collision dissociation (HCD) product ion-triggered electron transfer dissociation (ETD) mass spectrometry for the analysis of N-linked glycoproteins. *J. Proteome Res.* **11**, 4517–4525
480. Riley, N. M., and Coon, J. J. (2018) The role of electron transfer dissociation in modern proteomics. *Anal. Chem.* **90**, 40–64
481. Macias, L. A., Santos, I. C., and Brodbelt, J. S. (2020) Ion activation methods for peptides and proteins. *Anal. Chem.* **92**, 227–251
482. Riley, N. M., Hebert, A. S., Westphall, M. S., and Coon, J. J. (2019) Capturing site-specific heterogeneity with large-scale N-glycoproteome analysis. *Nat. Commun.* **10**, 1–13
483. Yu, Q., Wang, B., Chen, Z., Urabe, G., Glover, M. S., Shi, X., Guo, L. W., Kent, K. C., and Li, L. (2017) Electron-transfer/higher-energy collision dissociation (EThcD)-enabled intact glycopeptide/glycoproteome characterization. *J. Am. Soc. Mass Spectrom.* **28**, 1751–1764
484. Cao, Q., Yu, Q., Liu, Y., Chen, Z., and Li, L. (2020) Signature-ion-triggered mass spectrometry approach enabled discovery of N- and O-linked glycosylated neuropeptides in the Crustacean nervous system. *J. Proteome Res.* **19**, 634–643
485. Marino, F., Bern, M., Mommen, G. P. M., Leney, A. C., van Gaans-van den Brink, J. A. M., Bonvin, A. M. J. J., Becker, C., van Els, C. A. C. M., and Heck, A. J. R. (2015) Extended O-GlcNAc on HLA class-I-bound peptides. *J. Am. Chem. Soc.* **137**, 10922–10925
486. Čaval, T., Zhu, J., and Heck, A. J. R. (2019) Simply extending the mass range in electron transfer higher energy collisional dissociation increases confidence in N-glycopeptide identification. *Anal. Chem.* **91**, 10401–10406
487. Lee, L. Y., Moh, E. S. X., Parker, B. L., Bern, M., Packer, N. H., and Thaysen-Andersen, M. (2016) Toward automated N-glycopeptide identification in glycoproteomics. *J. Proteome Res.* **15**, 3904–3915
488. Khatri, K., Pu, Y., Klein, J. A., Wei, J., Costello, C. E., Lin, C., and Zaia, J. (2018) Comparison of collisional and electron-based dissociation modes for middle-down analysis of multiply glycosylated peptides. *J. Am. Soc. Mass Spectrom.* **29**, 1075–1085
489. Zacchi, L. F., and Schulz, B. L. (2016) SWATH-MS Glycoproteomics reveals consequences of defects in the glycosylation machinery. *Mol. Cell. Proteomics* **15**, 2435–2447
490. Zacchi, L. F., and Schulz, B. L. (2019) *Methods in Molecular Biology*. Humana Press Inc, New York, NY: 191–202
491. Zhou, C., and Schulz, B. L. (2020) Glycopeptide variable window SWATH for improved Data Independent Acquisition glycoproteomics. *Anal. Biochem.* **597**, 113667
492. Ye, Z., Mao, Y., Clausen, H., and Vakhrushev, S. Y. (2019) Glyco-DIA: a method for quantitative O-glycoproteomics with in silico-boosted glycopeptide libraries. *Nat. Methods* **16**, 902–910
493. Liu, Y., Chen, J., Sethi, A., Li, Q. K., Chen, L., Collins, B., Gillet, L. C. J., Wollscheid, B., Zhang, H., and Aebersold, R. (2014) Glycoproteomic analysis of prostate cancer tissues by SWATH mass spectrometry discovers N-acylethanolamine acid amidase and protein tyrosine kinase 7 as signatures for tumor aggressiveness. *Mol. Cell. Proteomics* **13**, 1753–1768
494. Lin, C. H., Krisp, C., Packer, N. H., and Molloy, M. P. (2018) Development of a data independent acquisition mass spectrometry workflow to enable glycopeptide analysis without predefined glycan compositional knowledge. *J. Proteomics* **172**, 68–75
495. Nwosu, C. C., Strum, J. S., An, H. J., and Lebrilla, C. B. (2010) Enhanced detection and identification of glycopeptides in negative ion mode mass spectrometry. *Anal. Chem.* **82**, 9654–9662
496. Madsen, J. A., Ko, B. J., Xu, H., Iwashkiw, J. A., Robotham, S. A., Shaw, J. B., Feldman, M. F., and Brodbelt, J. S. (2013) Concurrent automated sequencing of the glycan and peptide portions of O-linked glycopeptide anions by ultraviolet photodissociation mass spectrometry. *Anal. Chem.* **85**, 9253–9261
497. Riley, N. M., Rush, M. J. P., Rose, C. M., Richards, A. L., Kwiecien, N. W., Bailey, D. J., Hebert, A. S., Westphall, M. S., and Coon, J. J. (2015) The negative mode proteome with activated ion negative electron transfer dissociation. *Mol. Cell. Proteomics* **14**, 2644–2660
498. Hersberger, K. E., and Hakansson, K. (2012) Characterization of O-sulfopeptides by negative ion mode tandem mass spectrometry: superior performance of negative ion electron capture dissociation. *Anal. Chem.* **84**, 6370–6377
499. Kuo, C. W., and Khoo, K. H. (2020) Strategic applications of negative-mode LC-MS/MS analyses to expedite confident mass spectrometry-based identification of multiple glycosylated peptides. *Anal. Chem.* **92**, 7612–7620
500. Bern, M., Kil, Y. J., and Becker, C. (2012) Byonic: advanced peptide and protein identification software. *Curr. Protoc. Bioinformatics*
501. Polasky, D. A., Yu, F., Teo, G. C., and Nesvizhskii, A. I. (2020) Fast and comprehensive N- and O-glycoproteomics analysis with MSFragger-glyco. *Nat. Methods* **17**, 1125–1132
502. Lu, L., Riley, N. M., Shortreed, M. R., Bertozzi, C. R., and Smith, L. M. (2020) O-pair search with MetaMorpheus for O-glycopeptide characterization. *Nat. Methods* **17**, 1133–1138
503. An, Z., Shu, Q., Lv, H., Shu, L., Wang, J., Yang, F., and Fu, Y. (2018) N-linked glycopeptide identification based on open mass spectral library search. *Biomed. Res. Int.* **2018**, 1564136
504. Klein, J., and Zaia, J. (2020) Relative retention time estimation improves N-glycopeptide identifications by LC-MS/MS. *J. Proteome Res.* **19**, 2113–2121
505. Pappireddi, N., Martin, L., and Wühr, M. (2019) A review on quantitative multiplexed proteomics. *ChemBioChem* **20**, 1210–1224
506. Smith, B. J., Martins-De-Souza, D., and Fioramonte, M. (2019). In: *Methods in Molecular Biology*, Humana Press Inc, New York, NY: 3–39
507. Zhang, Y., Yin, H., and Lu, H. (2012) Recent progress in quantitative glycoproteomics. *Glycoconj. J.* **29**, 249–258
508. Pap, A., Prakash, A., F. Medzihradszky, K., and Darula, Z. (2018) Assessing the reproducibility of an *O* -glycopeptide enrichment method with a novel software, Pinnacle. *Electrophoresis* **39**, 3142–3147
509. McAlister, G. C., Nusinow, D. P., Jedrychowski, M. P., Wühr, M., Huttlin, E. L., Erickson, B. K., Rad, R., Haas, W., and Gygi, S. P. (2014) MultiNotch MS3 enables accurate, sensitive, and multiplexed detection of differential expression across cancer cell line proteomes. *Anal. Chem.* **86**, 7150–7158
510. Wenger, C. D., Lee, M. V., Hebert, A. S., McAlister, G. C., Phanstiel, D. H., Westphall, M. S., and Coon, J. J. (2011) Gas-phase purification enables accurate, multiplexed proteome quantification with isobaric tagging. *Nat. Methods* **8**, 933–935
511. Zhu, H., Qiu, C., Gryniewicz-Ruzicka, C. M., Keire, D. A., and Ye, H. (2020) Multiplexed comparative analysis of intact glycopeptides using electron-transfer dissociation and synchronous precursor selection based triple-stage mass spectrometry. *Anal. Chem.* **92**, 7547–7555
512. Fang, P., Ji, Y., Silbern, I., Doebele, C., Ninov, M., Lenz, C., Oellerich, T., Pan, K.-T., and Urlaub, H. (2020) SugarQuant: a streamlined pipeline for multiplexed quantitative site-specific N-glycoproteomics. *Nat. Commun.* **11**, 5268