Review

Pharmaceutical BIOPROCESSING

Pharm. Bioprocess.
(2013) 1(1), 89–104

Deciphering O-glycomics for the development and production of biopharmaceuticals

The functional impact of glycosylation on drug efficacy and safety profiles has been demonstrated in a wide range of biopharmaceuticals. Understanding of the N-glycosylation pathway and the advent of analytical technologies has enabled both detailed and rapid characterization of N-glycans, thus providing critical insights for regulatory compliance. In comparison, O-glycosylation encompasses several types of protein modifications by a more heterogeneous pool of sugars, and the understanding of its biology is less mature. Concomitantly, there exist several limitations in O-glycan analytical strategies, impacting both O-glycoprotein-based drug development and regulatory compliance. Here, we aim to provide a critical review of the biology and functional importance of O-glycosylation in the context of existing and potential O-glycoprotein drugs, as well as the corresponding analytical methods. The ultimate goal is to identify the gaps in current analytical methods and propose potential future directions for O-glycosylation analysis, to support the development and production of O-glycosylated biopharmaceuticals.

Peiqing Zhang*[1‡], Tianhua Wang[1], Muriel Bardor[1,2] & Zhiwei Song[1‡]

[1]Bioprocessing Technology Institute, 20 Biopolis Way, #06-01 Centros, Singapore (138668)

[2]Université de Rouen, Laboratoire Glycobiologie et Matrice Extracellulaire Végétale EA 4358, Institut de Recherche et d'Innovation Biomédicale (IRIB), Faculté des Sciences, 76821 Mont-Saint-Aignan Cédex, France

*Author for correspondence:
Tel.: +65 6407 0940
Fax: +65 6478 9561
E-mail: zhang_peiqing@bti.a-star.edu.sg

‡Authors contributed equally

Protein glycosylation is the most common type of post-translational modification. It refers to the covalent attachment of a carbohydrate moiety to the polypeptide backbone. Two major types of glycosylation can be classified based on the site of attachment: in N-linked glycosylation, the glycan is attached to the side chain of an Asn residue located in the consensus sequence of Asn-X-Ser/Thr (note position 2 [X] can be any amino acid except Pro; occasionally, position 3 can be a Cys residue); in O-linked glycosylation, the glycan is attached to the side chain of a Ser or Thr residue. No consensus sequence has been identified for the major type of O-glycosylation, that is, mucin-type O-glycosylation [1].

A substantial portion of the protein-based pharmaceuticals on the market are glycoproteins. Various glycan structures can critically modulate the physicochemical properties of proteins, affect their *in vivo* stability, and have a functional impact on their therapeutic potentials, and in some cases can affect the safety profiles of such drugs [2–4].

The functional impact of N-glycans on monoclonal antibodies (mAb) is widely recognized. Human IgG1 isotype mAbs bear one N-linked oligosaccharide at Asn^{297} on each of the Fc (Fragment crystalizable) regions of heavy chains. These N-glycans can affect the interaction of the Fc portion with Fc receptors (FcγRs), which are responsible for antibody-dependent cell-mediated cytotoxicity (ADCC). It is now well established that the ADCC activity of such therapeutic mAbs can be greatly increased by reducing the level of fucosylation on the N-glycan of the antibody [5]. Additionally, the effect of glycosylation on *in vivo* bioactivity of drugs has been shown in the context of erythropoietin (EPO) [3]. It was found that the presence of N-acetylneuraminic acid (Neu5Ac), which is the human type of sialic acid (Sia) on EPO, a glycoprotein drug primarily used for the treatment of anemia resulting from kidney disease

10.4155/PBP.13.7 ISSN 2048-9145

Key Terms

O-glycosylation: Covalent attachment of a carbohydrate moiety to a serine or threonine residue via the oxygen atom of the hydroxyl side chain.

Glycosylation-associated critical quality attributes: Examples of well-defined glycosylation-associated critical quality attributes include the level of core fucosylation for IgG Fc N-glycans and the level of erythropoietin sialylation.

and cancer therapy, can prevent premature clearance of the protein by the liver asialoglycoprotein receptor. Hence, proper sialylation of EPO can lead to prolonged *in vivo* drug circulation and higher *in vivo* bioactivity [3,6]. Glycosylation can also affect the bioactivity of drugs through specific recognition by target cells. One example of this is glucocerebrosidase (GCase) for enzyme replacement therapy of Gaucher's disease. The efficacy of this therapy largely depends on the effective targeting and internalization of the GCase into macrophages, which is mediated by the terminal mannose (Man) residues on the N-glycans of GCase [7].

Where drug safety is concerned, the major mammalian nonhuman Sia N-glycolylneuraminic acid (Neu5Gc) is considered an undesired, aberrant form of sialylation for therapeutic glycoproteins as it can induce an immunogenic response [8]. Galα1,3-Gal (or αGal in short) is another well-known immunogenic sugar epitope. Anti-αGal IgE antibodies have been found in high levels in some individuals who developed hyperallergic reactions after being treated with recombinant mAbs (e.g., cetuximab) bearing αGal epitopes [9].

Therefore, the glycosylation pattern constitutes several critical quality attributes of recombinant therapeutic proteins. Ideally, a producing clone for recombinant production of glycoproteins should be selected based on the optimal glycosylation profile. This optimal profile should be maintained during the manufacturing and purification processes, so that drug substances produced in different batches will have consistent efficacy and similar risk if any. In general, mammalian cells, especially Chinese Hamster Ovary (CHO) cells, are more frequently used for producing glycoproteins. This is because glycosylation profiles of the drugs from such systems are more similar to humans than non-mammalian cells, including insect, plant and yeast systems [4]. However, abrupt changes in glycosylation pattern of biopharmaceuticals have been reported, possibly as a consequence of a manufacturing process change [10]. This is due to the fact that glycan structures on recombinant proteins are not directly encoded by the host genome, but instead can be affected by host genetic background and culture environment [11]. Thanks to understanding of the N-glycosylation biosynthetic pathway, various means aiming at optimizing and maintaining the consistent N-glycosylation profile of recombinant glycoproteins have been proposed with varied effects [4,12,13]. In comparison to N-glycosylation, our understanding of the effect of O-glycosylation on protein therapeutics is still rather limited despite several lines of evidence suggesting a context-dependent impact [14,15].

Due to the critical impact of glycosylation on drug safety and efficacy and its heterogeneity and dynamics, regulatory agencies around the world require drug manufacturers to both characterize and maintain the glycan profile of their products. For instance, the International Conference on Harmonization provides guidance on test procedures and acceptance criteria for biotechnological/biological products (International Conference on Harmonization Q6B) [16]. For glycoproteins, it states that carbohydrate content is to be determined while the glycan structure and site information should be analyzed to the greatest extent possible [16]. While the importance of glycan analysis needs to be emphasized, the guidance does not provide a universal set of acceptance criteria for the glycan profile of the drug since such criteria depend on the specific protein therapeutics in consideration and the respective acceptance limit is the intellectual property of the manufacturer [4].

In practice, biochemical testing for biopharmaceuticals usually takes place in two levels – lot release and characterization. In the context of glycan profile testing, while lot release is performed on each batch to check whether the glycosylation is similar to previous batches so as to ensure consistency in production [17], on the other hand characterization work is more thorough, but performed only on selected representative batches before licensure or major process changes to fully measure the carbohydrate structures on the glycoprotein [18].

Controlling the glycosylation of biopharmaceuticals is a complex process that requires measures beyond merely testing the product at the end of manufacturing. Recently, many regulatory agencies, including the US FDA and the European Medicines Agency, have proposed some methodological approaches to help biopharmaceutical manufacturers incorporate quality considerations into the design process via the quality-by-design (QbD) framework. The purpose of implementing QbD is to apply knowledge about the product into every step of the manufacturing process so that one can be assured of the product quality by carefully controlling the process [19].

For successful QbD implementation on the production of protein therapeutics with desired glycosylation patterns, one must first understand and identify the important features of the biopharmaceutical that support its clinical use for the particular indication, which is termed as the mechanism of action. In the case of mAb manufacturing, ADCC can be such an mechanism of action as this is how an antibody activates the immune response, which is a critical component in achieving its intended clinical use. The next level is to pinpoint the

impact glycan structures can have on such mechanisms of action and define these properties as glycosylation-associated critical quality attributes (gCQAs). As described previously, fucosylation of Fc N-glycan, sialytion of EPO N-glycans, and terminal Man of GCase can all be considered as gCQAs [20]. In contrast, O-glycan-related CQAs remain less clearly defined.

Apart from identification of the gCQAs based on existing knowledge in glycobiology, implementation of QbD also relies on the ability to control process parameters relevant to the gCQAs identified. The manufacturing process must, therefore, be assessed to locate those factors that can impact gCQAs while process analytical technology needs to be put in place to accurately measure the defined gCQAs at every stage of process design and operation, so as to ensure that the glycan profiles on the produced biopharmaceuticals meet the required safety and efficacy standards.

A range of analytical methods and platforms have been established that are catered to the gCQAs. Significant technological advancements have been achieved that allow for rapid, quantitative and higher throughput analysis of protein glycosylation. For a quantitative Sia assay, a high-throughput method has recently been established [21], showing much higher sensitivity and a shorter analysis time than the traditional thiobarbituric acid assay. Glycan profiling (mostly N-glycans) can be conducted by separation-based methods and mass spectrometry (MS). Separation-based analysis includes chromatography methods and electrophoresis techniques. To facilitate the detection of glycan species, the glycans are typically conjugated with fluorescent molecules such as 2-aminobenzamide [22]. The aromatic rings on these tags impart a certain degree of hydrophobicity on the glycan and, hence, make reverse-phase (RP) liquid chromatography separation feasible. Alternatively, when normal phase (NP) or hydrophilic-interaction liquid chromatography (HILIC) is applied, the hydroxyl groups of the glycans can be harnessed to achieve proper separation [6]. Porous graphitic carbon (PGC)-based chromatography is also adopted for glycan separation as this technology is noted for its good selectivities in isomeric structures and improved retention for those charged glycans [23]. Alternatively, label-free native N-glycans can be analyzed by high performance anion exchange chromatography coupled to pulsed amperometric detection (HPAEC-PAD) [24]. In addition, N-glycans labeled with 8-aminopyrene-1,3,6-trisulfonate can be separated by capillary electrophoresis and detected and quantified by a laser-induced fluorescence unit [25].

MS systems with soft ionizers, especially matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI), are typically used for glycan profiling. For MALDI-MS analysis of mammalian N-glycans, permethylation of the glycan sample is often applied to neutralize the acidic residues on the glycan to ensure uniform ionization efficiency between charged (Sia-containing) and neutral oligosaccharides so that relative quantification is possible [26,27].

While more rapid and sensitive methods are being developed, some effort is also being put in to streamline and automate the workflow of N-glycan analysis to increase the throughput and extend its application from process monitoring to medium/process development and clone selection [28].

O-glycosylation can be found on a wide range of biopharmaceuticals (see **Table 1** and [29] for a list of approved biopharmaceuticals). Functionally, O-glycosylation was found to impose critical impacts on drug immunogenicity [14], protein secretion [15] and protective immunity against cancer [30], and maintenance of normal development [30] and physiology [31]. Structurally, O-glycosylation leads to a more heterogeneous pool of oligosaccharides with different initiation sugars and linkages [32]. The existing regulatory framework and increasing evidence showing the critical involvement of O-glycosylation in drug performance and diseases will necessitate extensive O-glycosylation analysis. However, O-glycosylation analysis is more challenging and less

Table 1. Sub-types of O-glycosylation, protein sequence features for O-glycan attachment and examples of modified proteins.

Types of O-glycosylation	Consensus sequence for glycan attachment	Examples of modified proteins
O-GalNacylation	Unknown	MUC1, EPO†, Enbrel†, G-CSF†
O-Fucosylation	$C^2X_{4-5}(S/T)C^3$ within EGF-like domain	Notch1, Factor VII†, Factor IX†, Factor XII, tissue plasminogen activator†
	$C^1X_{2-3}(S/T)C^2$ within TSR1 domain	Thrombospondin1 and 2, ADAMTS1-20
O-Glucosylation	C^1XSXPC^2 within EGF-like domain	Factor VII†, Factor IX†, Notch1
O-Mannosylation	Unknown	α-DG

†Indicates a protein with approved therapeutic application.

Key Terms

O-fucosylation: Two pathways exist for O-fucosylation of EGF-like domain and TSR1 domain, initiated by POFUT1 and POFUT2, respectively.

α-dystroglycan: Membrane protein that is translated as a single polypeptide and later cleaved into two subunits, a distal α-subunit and a membrane-spanning β-subunit. The α-subunit contains four N-glycans and a mucin-like domain with multiple O-GalNAc and O-Man glycans.

Enbrel®: Homodimeric TNF receptor II (TNFRII)-Fc fusion protein produced in CHO cells for the treatment of rheumatoid arthritis. Each chain carries two N-glycans and multiple mucin-type O-glycans in the TNFRII region.

developed compared with N-glycosylation. The focus of the discussion in the following section will then be placed on the current understanding of O-glycosylation, the available analytical methods and applications, as well as future perspectives.

O-glycosylation analysis & current gaps

» An overview on the biology of O-glycosylation

O-glycosylation encompasses a variety of modifications determined by the innermost (termed reducing end) monosaccharide. In the context of mucin-type O-glycosylation, this reducing end sugar is an N-acetylgalactosamine (GalNAc) residue that can be further modified by other sugar types including galactose (Gal), N-acetylglucosamine (GlcNAc), GalNAc, fucose (Fuc), and Sia (Neu5Ac and/or Neu5Gc). In addition to GalNAc, other types of monosaccharides can also be O-linked to the protein backbones, such as Fuc, glucose (Glc) and Man **(Figure 1)** [1,33].

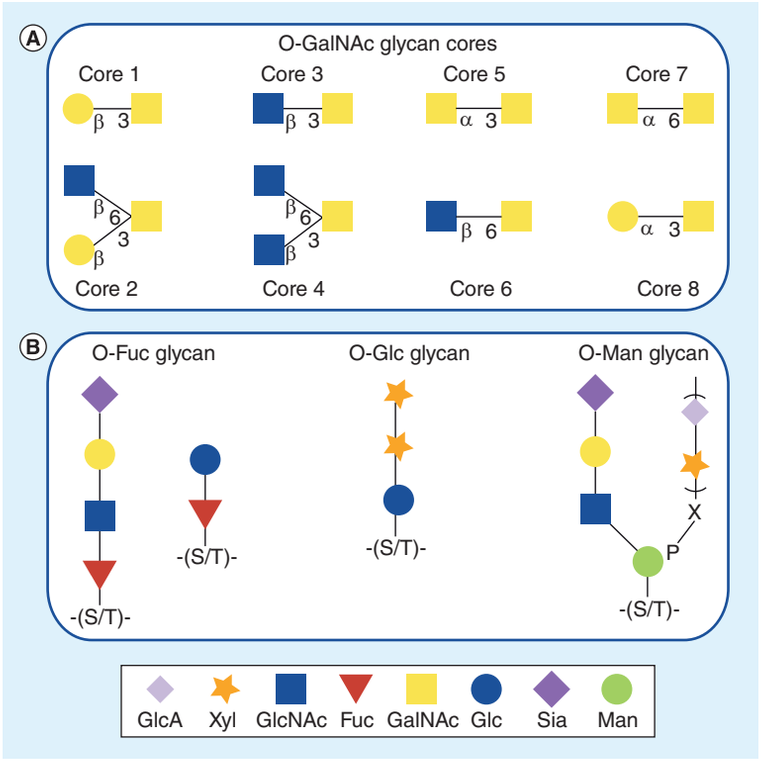

Figure 1. O-glycan structures. (A) Eight core structures of mucin-type O-glycosylation. All glycans were arranged such that the reducing ends face right. **(B)** Typical structures for O-Fuc glycan, O-Glc glycan and α-DG O-Man glycan. X in the O-Man glycan cartoon indicates an unknown sugar unit. Bracket indicates the repeats of Xyl-GlcA. Major references are [33,36].

Mucin-type O-glycosylation is initiated in the early Golgi compartment, catalyzed by GalNAc transferases that link a GalNAc from UDP-GalNAc to a Ser or Thr via the hydroxyl side chain. Existence of the consensus sequence for this modification is still unclear. In general terms, O-GalNAc glycosylation is thought to prefer regions rich in Ser/Thr, Pro and Ala [30] and several models have been proposed to predict such glycosylation sites [34,35]. The O-linked GalNAc can be further modified by Gal and GlcNAc in different linkages, generating eight core structures, each potentially subject to further elongation and substitution **(Figure 1)** [36].

Modification of Ser/Thr residues by a fucosyl glycan can be found in two distinct protein domains, namely the EGF-like repeat and thrombospondin type 1 repeat (TSR1) [37,38]. O-fucosylation is initiated in the endoplasmic reticulum (ER) by protein O-fucosyltransferases (POFUT). Two pathways were discovered that utilize POFUT1 and POFUT2 for the transfer of GDP-Fuc to a Ser or Thr hydroxyl side chain in EGF-like domain and TSR1 domain, respectively [39]. Specifically, the consensus sequence for O-fucosylation in the EGF-like domain is $C^2X_{4-5}(S/T)C^3$, where C^2 and C^3 refer to the second and third conserved Cys residues; which are separated by 4–5 amino acid residues ahead of a Ser/Thr residue. The consensus sequence for TSR1 type of O-fucosylation was found to be $C^1X_{2-3}(S/T)C^2$, where C^1 and C^2 refer to the first and second conserved Cys in the TSR1 domain. Subsequent modifications of the O-Fuc further diversify the two pathways, leading to the formation of typically Siaα2,3/6-Galβ1,4-GlcNAcβ1,3-Fuc-O-(Ser/Thr) tetrasaccharide in the context of EGF-like domain and Glcβ1,3-Fuc-O-(Ser/Thr) disaccharide in TSR1 domain **(Figure 1)** [39].

O-glucosylation is a rare type of modification, typically found in proximity to the EGF-like type of O-Fuc glycans on a small number of proteins with the consensus sequence of C^1XSXPC^2. This modification starts in the ER with the attachment of a Glc by a protein O-glucosyltransferase. The O-Glc can be extended by two xylose (Xyl) residues, forming a Xylβ1,3-Xylβ1,3-Glc-O-(Ser) trisaccharide **(Figure 1)**. With the cloning of the xylosyltransferase that adds the second Xyl to the O-Glc glycan, all the glycosyltransferases have been identified for this type of O-glycosylation [40].

O-mannosylation is another rare type of post-translational modification. However, it is abundantly present in the brain, giving rise to about 30% of the O-glycan pool [31]. This modification begins in the ER with the attachment of a Man to a Ser/Thr by protein O-mannosyltransferase 1 and 2. The O-Man is further

extended in the Golgi by a range of glycosyltransferases, including N-acetylglucosaminyltransferases, galactosyltransferases, and sialyltransferases. Additionally, several other Golgi-localized proteins were shown to be involved in the proper O-mannosylation of proteins, including Fukutin, Fukutin-related protein and LARGE, although their specificities need to be clearly elucidated [31]. Recently, O-mannosylation is gaining more and more attention due to its critical involvement in muscular dystrophy in the context of α-dystroglycan (α-DG). Mutations of the O-mannosylation-related glycosyltransferases result in improper glycosylation of α-DG, which abolishes its binding to laminin, leading to a class of inherited diseases called congenital muscular dystrophy [41]. O-Man glycans display a wide range of heterogeneous structures. The major structure found on α-DG is a Siaα2,3-Galβ1,4-GlcNAcβ1,2-Man-O-(Ser/Thr) tetrasaccharide **(Figure 1)** [31]. Recent structural analysis revealed α-DG O-Man glycans can be substituted at position 6 of the reducing end Man by a phosphoester [42], which is linked to an unknown sugar unit, which is in turn extended by repeating units of xylose and glucoronic acid (GlcA) in the form of -Xylα1,3-GlcAβ1,3- **(Figure 1)** [43]. Such extension was found to be dependent on the activity of LARGE and critically impacted the binding of α-DG to laminin [43]. Clearly, in-depth understanding of α-DG glycosylation, including O-mannosylation, is necessary for development of therapeutic agents against congenital muscular dystrophy [44].

The aforementioned types of O-glycosylation also exist in recombinant glycoproteins that find therapeutic applications in humans. In fact, two blockbuster biopharmaceutical drugs, recombinant human EPO and Enbrel® (a TNF receptor II-Fc fusion protein), are decorated with O-GalNAc glycans. Several coagulation blood factors are modified by O-Fuc and O-Glc glycans in their EGF-like domain, including recombinant Factor VII produced in BHK cells (brand name NovoSeven®) and recombinant Factor IX produced in CHO cells (brand name Benefix®) **(Table 1)** [29].

Similar to N-glycosylation, O-glycosylation is postulated to have general protective functions for the underlying proteins, including increasing the solubility and resistance to proteases. Importantly, a change of mucin-type O-glycosylation is observed in many types of solid tumors [30]. In fact, O-linked GalNAc (Tn antigen), Galα1,3-GalNAc (T antigen) and Siaα2,6-GalNAc (STn antigen) are found to be abundantly expressed in adenocarcinomas, and thus are termed tumor-associated antigens. These truncated O-glycans were found to be immunogenic in humans, especially when conjugated to MUC1 peptide backbones [45], thus raising the possibility of using the 'cancer-like' MUC1 glycopeptides as cancer vaccines [30]. In addition, O-glycosylation can also exhibit context-dependent functions. For example, O-fucosylation of Notch 1 was shown to be required for Notch signaling [46,47], whereas O-fucosylation of Factor VII seems dispensable from the binding of its EGF-like domain to tissue factor [48], but important for protein secretion [15].

The majority of the biopharmaceutical drugs are monoclonal antibodies of IgG isotype [29]. Except for isolated cases [49] these IgG molecules are not O-glycosylated, with only one N-glycan located in each Fc domain. However, it is already apparent that O-glycosylation is prevalent in many other classes of therapeutic proteins **(Table 1)**. In-depth understanding of the O-glycosylation status of these drug substances will shed light on the structure–function relationship of the O-linked sugars, which may lead to the identification of functionally favorable O-glycan structures to improve drug efficacy and safety profile. Even without a detailed understanding of the O-glycan function, the risk-based approach for regulatory approval still warrants the elucidation of the O-glycosylation pattern in a routine and quantitative manner. This is especially important for maintaining a consistent product quality profile for both innovator drugs and their follow-on products (or, biosimilars) to minimize the potential drifting in efficacy and safety profile. Additionally, research in alternative expression systems for glycoprotein production has been an ongoing effort. Tremendous progress has been made over the past few years, leading to the humanization, albeit to various extents, of N- and O- glycosylation in non-animal cell lines ([50,51] and reviewed in [6]). This field is further catalyzed by the recent FDA approval of taliglucerase, the first plant-derived recombinant glycoprotein drug [52]. Again, feasibility of these alternative platforms will, in part, depend on the O-glycan structures if they are intended for the production of proteins bearing 'human-like' O-glycans.

However, the knowledge of O-glycosylation biology is less mature compared with N-glycosylation. First, the consensus sequence of the O-glycan attachment site is still unknown, making O-glycosylation less predictive. Second, biosynthesis of O-glycans requires a more heterogeneous collection of glycosyltransferases. For example, unlike N-glycans that share a single Man3GlcNAc2 core structure, there exist eight core structures for mucin-type O-glycans, each formed by the catalytic activities of different glycosyltransferases. Furthermore, for the proximal GalNAc alone, there exist at least 20 GalNAc transferases in humans that are variably expressed in different tissues [53]. Thus, the O-glycome is largely shaped by the tissue- and cell line-dependent expression pattern of the related glycosyltransferases. Gill *et al.* showed the

striking GalNAc transferase relocation from Golgi to ER in response to EGF stimulation, and this relocation resulted in remodeling of cell surface and mucin O-glycosylation [54]. This provided strong evidence that O-glycosylation can be influenced by environmental cues such as growth factors existing in the cell culture medium. Third, analytical strategies for O-glycosylation are less well developed compared with N-glycosylation, thus forming a negative feedback loop with the inadequate understanding of its biology. These factors limit our understanding about the structure–function relation of O-glycosylation and impede the discovery of new drugs and drug targets, and therefore represent major hurdles for the development of novel and follow-on biopharmaceutical protein-bearing O-glycans. Below we provide a brief review of current technologies for O-glycosylation analysis that can be used for in-depth characterization and routine monitoring of recombinant protein O-glycosylation.

» Structural analysis of O-glycosylation

There are two complementary O-glycosylation analysis workflows. In the first approach, O-glycans are released from glycoproteins and subjected to labeling and/or derivatization before being analyzed by separation-based or MS methods. In the second approach, peptides carrying O-glycans (O-glycopeptides) are released from intact proteins and analyzed as a single entity in the first stage, usually by an LC–MS system, before being subjected to fragmentation to sequence both the peptides and the attached O-glycans by MS/MS.

Release of O-glycans

Release of N-glycans is typically achieved by PNGase F (or PNGase A in the case of plant-derived N-glycans), resulting in an intact reducing end for tagging. In addition, the mild enzymatic treatment preserves the structural integrity of N-glycans. In contrast, the specificities of the reported O-glycanase are predominantly restricted to T antigen (Galβ1,3-GalNAcα-Ser/Thr) [55,56]. Despite isolated reports describing O-glycanases of broader specificities [57], a universal O-glycanase that is capable of liberating O-glycans with more sophisticated branching and elongation is still unknown. Consequently, the application of the most established *Streptococcus pneumoniae* O-glycanase is rather limited. A prerequisite is pre-treatment using sialidase and other necessary exo-glycosidases to reveal the T antigen core in order for the O-glycanase to function. Identification of a universal O-glycanase has been an ongoing effort in recent decades. Although there is still no indication of success at present and even in the near future, it is possible that the genome sequencing of an ever growing list of species and the advent of large-scale, screening-based protein engineering methods may lead to the generation of a universal O-glycanase by manipulating the existing O-glycanases.

Chemical release of O-glycans is typically conducted at alkaline conditions. At elevated pH, the released O-glycans are susceptible to degradation by losing the reducing end sugars, an effect termed 'peeling', thus affecting the overall structural integrity. To control this problem to a minimal extent, a commonly used approach is to concomitantly reduce the released O-glycans by converting the reducing end GalNAc to GalNAcitol using reducing agents such as $NaBH_4$. Such a reductive elimination method has been shown to be most reliable in preserving the O-glycan structures. However, one serious drawback is that the reduced sugar prevents further glycan labeling through the reducing end hydroxyl group and abolishes the possibility of utilizing this end for fluorescence-based detection and quantitation. Although a label-free detection method, such as HPAEC-PAD, can provide sensitive quantitation without the need of a fluorophore [58], its application in native, label-free O-glycan analysis is still less well developed compared with the analysis of N-glycans, due to the lack of a comprehensive O-glycan standard library for peak identification.

In order to minimize the peeling effect in the alkaline elimination conditions, weak bases have been tested. The use of ammonium [59], dimethylamine [60] and ammonium carbamate [61], have been shown to suppress, but not totally avoid, the peeling effect. Additionally, the β-elimination can be accelerated by a microwave device, which is often employed in nonspecific proteolytic digestion of glycopeptides. In a parallel approach to shorten the reaction time, Yamada *et al.* developed an O-glycan release device, in which O-glycans were released by LiOH at 60°C for only 0.7 min at a continuous flow setting [62]. The reaction mixture containing released O-glycans is immediately neutralized by a cation exchange resin, minimizing peeling to a negligible level. This flow-based release method was further hyphenated to an MS system, allowing for automated O-glycan release and analysis [63]. The major determinant of this promising method lies in the availability of the instrumentation. It is reasonable to postulate that the instrument and method can be applied to the release of other types of O-glycans, but this remains to be tested.

Hydrazinolysis is another nonreductive method for O-glycan release without excessive peeling. Recently, Kozak *et al.* showed that the peeling effect could be suppressed by the addition of a tiny amount of trifluoroacetic acid (0.1% v/v) or EDTA (mM range) prior to hydrazinolysis, possibly by chelating cations [64]. However, the involvement of highly hazardous hydrazine

still represents a major limitation of this method. Additionally, all chemical release methods lack the required specificity for the selective release of O-glycans instead of N-glycans.

Therefore, the current gap in O-glycan release mainly lies in the lack of a robust and efficient method that liberates the O-linked oligosaccharides with an intact reducing end and structural integrity. It is noteworthy that endoglycosidases for releasing intact O-fucosylated, O-glucosylated and O-mannosylated glycans are completely unknown. The availability of a universal O-glycanase or a mild, yet robust method has twofold additional advantages:

- It preserves the underlying protein/peptide, thus paving the way for O-glycosylation site identification as well as functional analysis;
- It will enable the specific release of a particular type of O-glycan (e.g., O-Man glycans instead of all O-glycan species), thus allowing for targeted structural analysis.

Structural analysis of the released O-glycans

Similar to N-glycans, the released O-glycans can be analyzed by chromatography methods based on elution profile, or by MS methods, or both in the form of LC–MS. A major consideration for choosing a suitable analytical method depends on the treatment done to the O-glycans. This is because the physicochemical properties of the O-glycans after treatment affect their interaction with chromatographic stationary phases, as well as the preferred ion mode under specific MS settings.

O-glycan analysis by LC

Native O-glycans can be separated by either NP [65] or RP [66] columns in a complementary manner. More recently, HILIC [67] and PGC columns [68] have been applied to glycan separation. HILIC-based glycan analysis was shown to be a straight forward method with the ability to resolve some isomeric glycan structures. The elution time of a glycan species can be reported in terms of glucose units, which can provide valuable structural information on the glycan by referencing to a dextran ladder and online databases, most notably the GlycoBase [201], of glycan structures with known glucose units values [66]. The advent of ultra-performance liquid chromatography (UPLC) based on sub-2 µm beads dramatically shortens the analysis time and improves the peak resolution, as demonstrated by HILIC-UPLC analysis of IgG N-glycans [69]. A PGC column can be operated over a wider pH range and was shown to have higher resolving power towards more structural isomers [68]. A technical note is that both N- and O-glycans are preferably reduced prior to PGC separation to eliminate the α/β-isomerization of the reducing end hydroxyl group to simplify the analysis [68]. PGC as a relatively new technology is less applied in glycan analysis of biopharmaceutical glycoproteins (primarily IgGs). A recent report compared HILIC, RP and PGC stationary phases and suggested the lack of reproducibility and robustness of PGC in glycan analysis [70]. Obviously, these issues have to be resolved in order for PGC-based glycan analysis to be readily acceptable for the regulatory submission of biopharmaceutical drugs. In recent years, technology developments in microfluidics have given rise to chip-based separation devices with HILIC [71], RP [72] or PGC stationary phases [73,74]. These chip-based HPLC separation devices are often coupled to a downstream MS, allowing for structural confirmation of the glycan species, and additionally, upstream chips for glycoprotein digestion, glycan release and purification [74]. However, such a rapid (within 10 min), fully automated chip-based glycan analysis workflow was only demonstrated in the context of IgG N-glycans [74]. The application of such a platform for O-glycan analysis remains to be tested.

Detection and quantitation of separated O-glycan species in a chromatography setting is typically achieved optically or electrochemically. Although native glycans can be detected by absorbance at around 210 nm wavelength [75], glycans are preferably labeled with a fluorescent tag to increase the sensitivity of detection [76]. The most commonly used tag is 2-aminobenzamide [22]. Knezevic *et al.* reported a multiplexed labeling strategy using 2-aminobenzamide, aniline and 2-aminoacridone [77]. The three fluorescent dyes were shown to have minimal spectral overlap, thus allowing for three-channel data output from a single UPLC separation of plasma N-glycan samples. As for most UPLC-based glycan analytical methods without MS coupling, the identity of the glycan species usually requires exoglycosidase-based sequencing. Therefore, the multiplexed, UPLC-based method can be applied to O-glycan analysis, provided a comprehensive panel of O-glycan specific exoglycosidases is readily available.

Reduced O-glycan alditols in their native form can be analyzed by HPAEC-PAD, allowing for label-free detection. This is illustrated by the quantitative analysis of yeast O-Man glycans [58]. Alternatively, NP or RP chromatography can be employed with UV detection at around 210 nm wavelength, or coupled to an MS for detection. Permethylated O-glycans can be separated by reserved-phase chromatography and analyzed by MS.

O-glycan analysis by MS

MS can be a standalone system for glycan analysis without coupling to an upstream separation phase. In this case, the released O-glycans, derivatized or in their na-

tive forms, will be analyzed as a heterogeneous pool of structures. MALDI-MS and ESI-MS are the two most popular MS configurations for O-glycan analysis. In fact, an international multicenter report showed the two most reliable O-glycan analytical methods, namely, direct MS analysis of permethylated reduced O-glycan pools in positive ion mode of MALDI-MS and LC–MS analysis of native reduced O-glycans in negative mode [78]. Owing to the relatively higher salt tolerance and speed, MALDI-MS is a particularly efficient method for initial profiling of O-glycans. However, under a MALDI setting, neutral glycans are efficiently ionized in the positive ion mode while negatively charged glycans (sialylated or sulfated) are efficiently ionized in the negative ion mode. Therefore, the O-glycans are preferably permethylated allowing analysis of all structures under positive mode. Under such conditions, glycan species form singly charged sodium ion adducts, thus facilitating spectral interpretation. However, MALDI-MS relies on a matrix for efficient energy transfer for the ionization of glycan species. The most commonly used matrix, 2,5-dihydroxybenzoic acid, produces background peaks ranging up to approximately 500–700 *m/z*. This range covers the *m/z* values of several small O-glycan species. For example, a singly charged sodiated ion of the permethylated Tn antigen is 330.2 (*m/z*), whereas that of the T antigen is about 534.3 and the STn is about 691.4. These small ions can be masked by the high level of ions generated from the matrix. In contrast, ESI-MS typically produces background ions below 200 *m/z*, thus enabling sensitive detection of small O-glycan species. It is for this reason the authors favor the ESI-MS over MALDI-MS for analyzing samples containing such tumor-associated O-glycans. Permethylated O-glycan alditols can be directly infused into ESI-MS [79]. Because of structural heterogeneity, O-glycans directly sprayed into ESI-MS often have to be subjected to MS/MS for unambiguous structural assignment. The advantage of performing permethylation to the O-glycans is that it gives rise to a predictable fragmentation pattern of the O-glycan species, thus aiding the assignment of precursor structures. However, the permethylation reaction can potentially destroy several types of sugar modifications such as sulfation. The recent CHO-K1 genome sequencing study showed that CHO-K1 cells do not express sulfotransferases [80]. Therefore, CHO-K1-derived O-glycans are expected to be free of a sulfate group and permethylation is safe in this regard. However, it should be noted that previous studies reported the sulfation of CHO-derived O-glycans [81], possibly due to the activation of the sulfotransferase genes in the particular cell line. This highlights the importance of performing glycomic analysis during the clonal selection step of recombinant production (see below).

O-glycan analysis by LC–MS

Compared to direct infusion of permethylated O-glycan alditols, coupling of an ESI-MS to an upstream LC (especially nanoLC) has several advantages [32,76,82]. First, the LC dimension can separate certain structural isomers, enabling downstream unambiguous structural assignment by MS. Second, the nanoLC can serve as a trapping and concentration step, which increases the concentration of O-glycan species, hence significantly improving the sensitivity of detection. Third, an inherent problem of MS is the suppression of low-abundance ions. By segregating the O-glycan species, the chance of a low-abundance ion being suppressed by a high-abundance ion is reduced and this will lead to enhanced detection of minor O-glycan species. Additionally, the authors' experience is that permethylation of O-glycans reduces their stickiness and produces a more stable spray. Apart from LC–MS analysis of permethylated O-glycan alditols using a RP stationary phase, native O-glycan alditols can be analyzed by HILIC-LC–MS or PGC-LC–MS [32].

High-throughput O-glycan analysis

LC–ESI-MS^n of native O-glycans or permethylated O-glycan alditols is able to provide in-depth structural information, representing the current state-of-the-art O-glycan analytical method. A potential direction is the full automation of the whole analytical workflow ranging from O-glycan release, preparation and data acquisition to chromatogram/spectrum interpretation, which has been impressively developed for N-glycan analysis [74,83]. This will allow detailed, high-throughput O-glycan analysis for the selection of O-glycosylation-optimized host clones and routine monitoring of product quality. As previously described, the major hurdle impeding the HPLC/UPLC-based O-glycan analysis lies in the lack of a reliable and efficient release method for fluorescent tagging, a limited exoglycosidase library for O-glycan sequencing, and a still growing O-glycan structure database with critical information for glycan annotation based on elution profile [66], whereas the key for an LC–MS-based workflow is the ability to separate as many structure isomers by the LC dimension, and the sensitive detection of intact O-glycan ions and their corresponding fragment ions by MS/MS. The ability to generate cross-ring fragments will be particularly useful as it can yield information on the composition, as well as glycosidic linkage. Various studies have demonstrated such capability using different dissociation methods and negative or positive ion modes [84–86]. Ion mobility spectrometry (IMS) is a complementary ap-

proach to LC. IMS separates modules based on their shapes and, thus, represents an appealing technique for separating glycan structural isomers. Indeed, IMS-MS has been successfully applied to distinguish different Man7 structural isomers [87]. It has been also applied to medium-scale (~100 samples) studies on the serum glycan change in esophageal adenocarcinoma [88], as well as liver diseases [89]. The ability of IMS to segregate glycan ions of different charge states may be particularly beneficial for dissociation methods requiring a specific charge state, such as electron capture dissociation [90]. As a consequence of such properties, IMS has been demonstrated to be an attractive enhancer/sensitizer device that allows the detection of N-glycan species from post-PNGaseF treatment mixtures [91]. It is tempting to test the power of IMS hyphenated to MS/MS in high-throughput O-glycan analysis by virtue of its ability to separate isomeric structures, filter background noise and group glycan ions of different charge states.

Structural analysis of O-glycopeptides

Glycoproteomic analysis offers rich information about protein glycosylation in a site-specific manner. By analyzing a glycosylated peptide as a single analyte, the glycoproteomic approach yields information on both the peptide and the attached glycan. However, it represents the most challenging aspect in glyco-analytics with combinatorial complexity caused by the variable peptide sequence and structural heterogeneity of the attached glycans. A prominent example for such technical difficulty is the inability of current analytical methods to perform site-specific O-glycosylation analysis on mucin domains, particularly tandem repeats of such domains. Analysis of intact glycopeptides is primarily performed using LC–MS/MS (MS^n) in which the peptides and glycans are sequenced at different MS stages. Such analysis using different LC–MS instrumentations on different types of glycopeptide samples has been extensively reviewed by experts in the field [26,92–94]. Here we will only highlight a few key points in the analytical workflow and some recent examples of site-specific O-glycosylation analysis.

The LC–MS-based analysis can be affected by the source of glycopeptides. Complex mixtures of glycoproteins such as whole cell lysates and tissue homogenates contain a myriad of proteins that can be modified by a heterogeneous set of glycans at each site. At present, the majority of samples are either chemically synthesized glycopeptides, or glycopeptides enzymatically derived from purified proteins or lectin affinity-enriched proteins [94]. Lectin-affinity enrichment is a particularly useful technique in sample preparation for biomarker discovery [92] or the identification of glycosylation sites bearing pre-targeted glycan structures.

One common specific protease for cleaving the intact glycoproteins is trypsin, which cuts at the carboxyl side of Arg/Lys unless they are followed by Pro. However, tryptic digestion of proteins often results in peptides of various lengths, potentially generating glycopeptides of unfavorable lengths containing multiple glycans, which may complicate the analysis. Mucin-type O-glycosylation sites are typically found in tandem repeating units rich in Ser/Thr, Ala and Pro [30], and thus are considered resistant to tryptic digestion. Alternatively, nonspecific proteases such as pronase E and proteinase K have been applied. Pronase generates small peptides of 2–8 amino acid residues resulting in facile site-specific glycosylation analysis of high coverage [95,96]. Proteinase K has been used in combination with trypsin to release O-glycopeptides from human fibrinogen [97]. Immobilization of the proteases on beads is an appealing approach to achieve high reproducibility, high catalytic efficiency and reusability [98].

An LC dimension of separation after digestion of the glycoprotein is beneficial: it enriches the often low-abundance glycopeptides, thereby increasing the sensitivity of detection; fractionation of glycopeptides into different pools also reduces the overall heterogeneity in each subpopulation, and therefore reduces ion suppression and meanwhile simplifies the mass spectral interpretation. Separation can be achieved using HILIC [99], RP [100], NP [96] or PGC chromatography [73].

Perhaps the most critical factor in MS/MS sequencing of the glycopeptides lies in the mode of dissociation. Two commonly used dissociation methods are collision-induced dissociation (CID) and electron-transfer dissociation (ETD). These two modes can give rise to dramatically different fragmentation patterns under different ionization methods [101,102]. Low energy CID of electrosprayed glycopeptides predominantly resulted in fragmentation of the glycan moiety. However, studies have shown that this technique does allow the identification of O-GalNAc and O-Fuc glycosylation sites (reviewed in [102]). On the other hand, ETD promotes the fragmentation of the peptide backbone and preserves the structural integrity of the attached glycan [101]. Therefore, CID and ETD represent two complementary techniques for yielding structural information on both the glycan and the peptide [102,103]. There are other emerging dissociation techniques that have been applied to the analysis of glycan and glycopeptides, such as electron-capture dissociation and electron-detachment dissociation [90]. However, these are less developed and the mechanisms are still controversial compared with CID and ETD.

A glycoproteomic approach can be of particular value in O-glycosylation analysis. Several types of O-glycosylation, such as O-fucosylation and

O-glucosylation are relatively simple, with only a few possible glycan structures. Therefore, glycopeptide signatures of proteins bearing such modifications are relatively less heterogeneous, making spectral interpretation and detailed analysis more straightforward. Additionally, analyzing the O-glycopeptide as a whole can circumvent the problems encountered in O-glycan release, including the peeling effect and potential loss of glycan modification during permethylation. Such an approach is exemplified by the analysis of O-fucosylated TSR1 glycopeptide using nanoESI Q-TOF [104] and O-fucosylated glycopeptides derived from EGF-like repeat of Notch 1 [105].

Recently, Harrison *et al.* performed the analysis of O-glycopeptides derived from recombinant mouse α-DG-Fc fusion protein expressed in HEK293T cells [106]. Using a combination of offline nanoLC–MALDI-MS and online nanoLC–ESI-MS, the authors identified 38 glycopeptides that were modified by either O-Man or O-GalNAc glycans. In addition, MS/MS in the forms of MALDI-TOF/TOF or ESI-MS/MS using CID generated comprehensive fragment patterns, revealing the underlying peptide sequences, sites of glycan attachment, as well as structures of the glycans. Therefore, this study represents a valuable guide for designing experiments for site-specific analysis of α-DG glycosylation. Information derived from such analysis will shed light on understanding the pathogenesis of muscular dystrophy and may provide functional evidence for efficacy of drugs targeting this class of diseases.

Potential areas for further development

The impact of glycosylation on efficacy and safety profiles of biopharmaceutical proteins has been increasingly recognized. In light of the critical functional impact, glycosylation has to be maintained at a consistent and preferably optimal level [4]. However, this type of post-translational modification is not directly encoded in the genome of the host expression platforms. Control of glycosylation pattern thus requires a detailed understanding of the biology and a reliable analytical system for structural characterization and routine monitoring. This will allow the identification of gCQAs and constant surveillance of these gCQAs for effective QbD implementation. There are existing analytical methods, such as CE, HPLC and MS, that have provided comprehensive information on N-glycosylation of biopharmaceutical drugs. Many such technologies have formed the glyco-analytics platforms for regulatory approval of biopharmaceutical glycoproteins, comprehensive analysis and batch monitoring [18]. However, there is still a pressing need for a more rigorous regulation of glycosylation patterns throughout drug discovery and manufacturing processes, especially in light of the findings that a change in the process could lead to an abrupt change in critical quality attributes of the proteins, including glycosylation [10]. Furthermore, there is already a sizable list of therapeutic proteins bearing various types of O-glycans (**Table 1** and [29]) and the list is expected to grow substantially given multiple trials of MUC1 O-glycopeptide-based cancer vaccines [30,107]. However, the structure–function relationship of O-glycosylation remains less well defined. Additionally, the existing analytical platforms are mainly catered for the characterization of N-glycans. Clearly, one possible future direction of the O-glycomics field is rapid, quantitative analysis of O-glycan structures and their attachment sites.

O-glycan release remains a bottleneck in the structural characterization of O-glycans. Despite the far cry from a universal O-glycanase, the availability of a robust release method that allows fluorescent tagging that meanwhile preserves the structural integrity will streamline the subsequent O-glycosylation analytical methods to follow those for N-glycosylation analysis.

Site-specific analysis is arguably more challenging for O-glycosylation than N-glycosylation. This is in part due to the lack of a clearly defined consensus sequence for O-GalNAc glycan attachment. Additionally, expression of the GalNAc transferases for initiation of each O-GalNAc glycan has been shown to be dependent on the cell type and dynamically regulated by extracellular signals [53]. Therefore, site-specific O-glycosylation will be necessary, especially if alternative expression systems will be applied. The advent of LC–MS, and notably the PGC-LC–MS, has the potential to revolutionize the glyco-analytics field due to its superior resolving power and ability to automate the workflow. Indeed, IgG N-glycan characterization has been fully automated on such a chip-LC–MS system [74]. However, more applications need to be demonstrated for automated preparation and analysis of O-glycopeptides, ranging from purification, protein cleavage, and glycopeptide enrichment and separation, to MS/MS sequencing of both the peptide and the O-glycan.

Apart from the detailed characterization of a purified biopharmaceutical substance from cell culture, another important aspect of glycosylation analysis are the host cells. Because glycosylation of the recombinant product is largely controlled by the metabolism of the host cells, the glycosylation pattern of the host in many aspects can be predictive for that of the recombinant product. Previously we reported the generation of a collection of CHO glycosylation mutants, the CHO-gmt cells, and their applications in recombinant production of glycoproteins with homogeneous glycan structures [108]. The selection of such a glycosylation-optimized host increases the resilience of cell lines to process variations, allowing more ro-

bust control of the glycosylation profile. The development of targeted genetic manipulation approaches, such as zinc-finger nuclease technology, has enabled the generation of cell lines with tailored glycosylation attributes [109–111]. In addition, Davies *et al.* demonstrated that heritable traits, including the glycosylation pattern, can be selected from a wild-type CHO cell population [112], forming the basis for subcloning of glycosylation-optimized stable producing cell lines for second-generation biopharmaceuticals. Although orthogonal methods in theory can allow the selection of cell lines with pre-targeted glycosylation profiles, MS-based structural analysis can offer unparalleled depth of information. However, high-throughput glycomic/glycoproteomic analysis based on MS systems has not been demonstrated at the whole cell level. A major technical challenge is the heterogeneity of glycan/glycopeptide samples derived from whole cell lysates, which are intrinsically highly diverse and may contain high background caused by irrelevant cellular constituents. In this regard, the IMS may represent a particularly interesting technology for separating the meaningful signal from background noise [88,89]. Indeed, Harvey *et al.* demonstrated that coupling an IMS upstream of an MS dramatically improves the signal-to-noise ratio, allowing the detection of N-glycans in the post-PNGaseF treatment mixture [91]. It will be interesting to apply the IMS-MS system to the analysis of O-glycosylation in complex matrices such as whole cell lysates for the rapid identification of cells with favorable O-glycosylation profiles.

Finally, the recently published genome and transcriptome data of an ancestral CHO-K1 represents a rich resource for understanding the expression of each component in the O-glycosylation machinery [80]. By ruling out impossible biosynthetic pathways characterized by non-expression of the corresponding genes, this will prove useful for the structural assignment of O-glycans. Conversely, glycomic data can shed light on functions of putative glycosylation-related genes. Taken together, breakthrough in the O-glycomics fieldwill form a positive feedback loop with the functional O-glycobiology. Together, this will enable the discovery of drugs and drug targets bearing O-glycosylation and, subsequently, empower us to effectively control the O-glycosylation profile for the production of biopharmaceutical proteins with consistent quality.

Future perspective

With the advancements in O-glycosylation analysis and the increasing understanding of its role in health and disease, the list of O-glycosylated protein drugs and drug candidates is expected to grow tremendously. The success of such therapeutic agents will largely depend on

Executive summary
Background
» Glycosylation has a profound impact on safety and efficacy of biopharmaceutical drugs.
» Regulatory guidelines are in place requesting extensive and routine analysis of glycosylation profile.
» Current analytical strategies are mainly catered to N-glycosylation.
O-glycosylation analysis & current gaps
» We provide an overview on the biology of O-glycosylation.
» The biology of O-glycosylation is less understood.
» O-glycosylation comes in different flavors and leads to high structural heterogeneity.
Structural analysis of O-glycosylation
» A robust enzymatic O-glycan release method is still missing.
» Released O-glycans can be labeled, derivatized or natively analyzed by HPLC, MS or LC–MS with different advantages and disadvantages.
» The concept of automated O-glycomics needs to be demonstrated in the context of more biopharmaceutical proteins.
» O-glycopeptide analysis based on MS or LC–MS yields more insightful information, but represents significant technological challenges.
» High-throughput O-glycosylation analysis remains to be developed by HLPC, MS or IMS–MS-based technologies.
Potential areas for further development
» O-glycopeptide-based molecules hold future promise for therapeutic applications and deserve in-depth analysis.
» A robust method is needed for faithful release of O-glycan for subsequent fluorescent tagging.
» High-throughput approaches should be extended to whole-cell glycomics and glycoproteomics for selection of favorable host cell lines.
» O-glycomics should be integrated with genomics and transcriptomics data to facilitate the understanding of O-glycobiology for drug development.
Future perspective
» Future development in the field will depend on the positive feedback loop between structural analysis by detailed and high-throughput technologies and the integration with other -omics data.

the demonstration of consistency in quality including O-glycosylation pattern, and the ability to effectively maintain the consistency during bioproduction stages. The future development of structural O-glycomics will probably go in two complementary directions: detailed O-glycan/O-glycopeptide analysis for fine structural information (including glycan composition, linkage, attached peptide sequences), as well as high-throughput O-glycan/O-glycoproteomic characterization for product and host cell line screening. The former direction will yield critical dataset for the latter, with information on elution profile, glycan footprint, fragmentation pattern and so forth. In this regard, what remains to be further developed also includes O-glycan standards for establishing structural databases that can be easily accessed and/or embedded into analytical instruments, and software for automated chromatogram/spectrum interpretation. Finally, the integration of O-glycomics with other -omics data (such as genomics, transcriptomics and metabolomics) will become an interesting area for the understanding of regulation of O-glycosylation at a systems level and may lead to effective targeting of O-glycosylation-related diseases and the production of O-glycosylation-optimized glycoprotein drugs.

Acknowledgements

The authors would like to thank Y Yang for critical review of the manuscript.

Financial & competing interests disclosure

*This work is supported by Biomedical Research Council, Agency of Science, Technology and Research (A*STAR), Singapore. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.*

No writing assistance was utilized in the production of this manuscript.

References

Papers of special note have been highlighted as:
- ■ of interest
- ■■ of considerable interest

1 Moremen KW, Tiemeyer M, Nairn AV. Vertebrate protein glycosylation: diversity, synthesis and function. *Nat. Rev. Mol. Cell Biol.* 13(7), 448–462 (2012).

2 Liu DTY. Glycoprotein pharmaceuticals – scientific and regulatory considerations, and the United States orphan drug-act. *Trends Biotechnol.* 10(4), 114–120 (1992).

3 Sinclair AM, Elliott S. Glycoengineering: the effect of glycosylation on the properties of therapeutic proteins. *J. Pharm. Sci.* 94(8), 1626–1635 (2005).

4 Hossler P, Khattak SF, Li ZJ. Optimal and consistent protein glycosylation in mammalian cell culture. *Glycobiology* 19(9), 936–949 (2009).

■ **Provides a comprehensive overview of approaches to control glycosylation pattern in cell culture processes.**

5 Peipp M, Van Bueren JJL, Schneider-Merck T *et al.* Antibody fucosylation differentially impacts cytotoxicity mediated by NK and PMN effector cells. *Blood* 112(6), 2390–2399 (2008).

6 Lingg N, Zhang P, Song Z, Bardor M. The sweet tooth of biopharmaceuticals: importance of recombinant protein glycosylation analysis. *Biotechnol. J.* 7(12), 1462–1472 (2012).

7 Weinreb NJ. Imiglucerase and its use for the treatment of Gaucher's disease. *Expert Opin. Pharmacother.* 9(11), 1987–2000 (2008).

8 Ghaderi D, Taylor RE, Padler-Karavani V, Diaz S, Varki A. Implications of the presence of N-glycolylneuraminic acid in recombinant therapeutic glycoproteins. *Nat. Biotechnol.* 28(8), U863–U145 (2010).

9 Chung CH, Mirakhur B, Chan E *et al.* Cetuximab-induced anaphylaxis and IgE specific for galactose-alpha-1,3-galactose. *N. Engl. J. Med.* 358(11), 1109–1117 (2008).

10 Schiestl M, Stangler T, Torella C, Cepeljnik T, Toll H, Grau R. Acceptable changes in quality attributes of glycosylated biopharmaceuticals. *Nat. Biotechnol.* 29(4), 310–312 (2011).

■ **Reported significant change in critical quality attributes of several major innovator biopharmaceuticals, possibly as a result of change in manufacturing mode.**

11 Hossler P. Protein glycosylation control in mammalian cell culture: past precedents and contemporary prospects. *Adv. Biochem. Eng. Biotechnol.* 127, 187–219 (2012).

12 Zhang P, Tan DL, Heng D *et al.* A functional analysis of N-glycosylation-related genes on sialylation of recombinant erythropoietin in six commonly used mammalian cell lines. *Metab. Eng.* 12(6), 526–536 (2010).

13 Goh JS, Zhang P, Chan KF, Lee MM, Lim SF, Song Z. RCA-I-resistant CHO mutant cells have dysfunctional GnT I and expression of normal GnT I in these mutants enhances sialylation of recombinant erythropoietin. *Metab. Eng.* 12(4), 360–368 (2010).

14 Hermeling S, Crommelin DJA, Schellekens H, Jiskoot W. Structure-immunogenicity relationships of therapeutic proteins. *Pharm. Res.* 21(6), 897–903 (2004).

15 Bolt G, Steenstrup TD, Kristensen C. All post-translational modifications except propeptide cleavage are required for optimal secretion of coagulation factor VII. *Thromb. Haemost.* 98(5), 988–997 (2007).

16 Department of Health and Human Services. International Conference on Harmonization; guidance on specifications: test procedures and acceptance criteria for biotechnological/

biological products. *FDA HHS. Fed. Regist.* 64(159), 44928–44935 (1999).

17 Siemiatkoski J, Ma S, Park J, Brorson K, Swann P, McLeod L. Glycosylation of therapeutic proteins. *BioProcess Int.* 9(6), 48–53 (2011).

18 Read EK, Park JT, Brorson KA. Industry and regulatory experience of the glycosylation of monoclonal antibodies. *Biotechnol. Appl. Biochem.* 58(4), 213–219 (2011).

19 Rathore AS, Winkle H. Quality by design for biopharmaceuticals. *Nat. Biotechnol.* 27(1), 26–34 (2009).

20 Del Val IJ, Kontoravdi C, Nagy JM. Towards the implementation of quality by design to the production of therapeutic monoclonal antibodies with desired glycosylation patterns. *Biotechnol. Prog.* 26(6), 1505–1527 (2010).

21 Markely LRA, Ong BT, Hoi KM, Teo G, Lu MY, Wang DIC. A high-throughput method for quantification of glycoprotein sialylation. *Anal. Biochem.* 407(1), 128–133 (2010).

22 Ruhaak LR, Zauner G, Huhn C, Bruggink C, Deelder AM, Wuhrer M. Glycan labeling strategies and their use in identification and quantification. *Anal. Bioanal. Chem.* 397(8), 3457–3481 (2010).

23 Melmer M, Stangler T, Premstaller A, Lindner W. Comparison of hydrophilic-interaction, reversed-phase and porous graphitic carbon chromatography for glycan analysis. *J. Chromatogr. A* 1218(1), 118–123 (2011).

24 Lee YC. Carbohydrate analyses with high-performance anion-exchange chromatography. *J. Chromatogr. A* 720(1–2), 137–149 (1996).

25 Mechref Y, Muzikar J, Novotny MV. Comprehensive assessment of N-glycans derived from a murine monoclonal antibody: a case for multimethodological approach. *Electrophoresis* 26(10), 2034–2046 (2005).

26 Mechref Y, Novotny MV. Structural investigations of glycoconjugates at high sensitivity. *Chem. Rev.* 102(2), 321–369 (2002).

27 Sheeley DM, Merrill BM, Taylor LCE. Characterization of monoclonal antibody glycosylation: comparison of expression systems and identification of terminal alpha-linked galactose. *Anal. Biochem.* 247(1), 102–110 (1997).

28 Reusch D, Haberger M, Selman MH *et al.* High-throughput work flow for IgG Fc-glycosylation analysis of biotechnological samples. *Anal. Biochem.* 432(2), 82–89 (2013).

29 Walsh G. Biopharmaceutical benchmarks 2010. *Nat. Biotechnol.* 28(9), 917–924 (2010).

30 Tarp MA, Clausen H. Mucin-type O-glycosylation and its potential use in drug and vaccine development. *Biochim. Biophys. Acta.* 1780(3), 546–563 (2008).

■ **Covers the biology of mucin-type O-glycosylation and strategies of utilizing O-glycosylated MUC1 molecules for vaccine development.**

31 Stalnaker SH, Stuart R, Wells L. Mammalian O-mannosylation: unsolved questions of structure/function. *Curr. Opin. Struct. Biol.* 21(5), 603–609 (2011).

32 Jensen PH, Kolarich D, Packer NH. Mucin-type O-glycosylation - putting the pieces together. *FEBS J.* 277(1), 81–94 (2010).

33 Freeze HH, Haltiwanger RS. Other classes of ER/Golgi-derived glycans. In: *Essentials of Glycobiology, (2nd edition).* Varki A, Cummings R, Esko J *et al.* (Eds). Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA (2009).

34 Julenius K, Molgaard A, Gupta R, Brunak S. Prediction, conservation analysis, and structural characterization of mammalian mucin-type O-glycosylation sites. *Glycobiology* 15(2), 153–164 (2005).

35 Zhou K, Ai C, Dong P, Fan X, Yang L. A novel model to predict O-glycosylation sites using a highly unbalanced dataset. *Glycoconj. J.* 29(7), 551–564 (2012).

36 Brockhausen I, Schachter H, Stanley P. O-GalNAc Glycans. In: *Essentials of Glycobiology (2nd edition).* Varki A, Cummings R, Esko J *et al.* (Eds). Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA (2009).

37 Leonhard-Melief C, Haltiwanger RS. O-fucosylation of thrombospondin type 1 repeats. *Methods Enzymol.* 480, 401–416 (2010).

38 Harris RJ, Spellman MW. O-linked fucose and other post-translational modifications unique to EGF modules. *Glycobiology* 3(3), 219–224 (1993).

39 Luo Y, Nita-Lazar A, Haltiwanger RS. Two distinct pathways for O-fucosylation of epidermal growth factor-like or thrombospondin type 1 repeats. *J. Biol. Chem.* 281(14), 9385–9392 (2006).

40 Sethi MK, Buettner FF, Ashikov A *et al.* Molecular cloning of a xylosyltransferase that transfers the second xylose to O-glucosylated epidermal growth factor repeats of notch. *J. Biol. Chem.* 287(4), 2739–2748 (2012).

41 Dobson CM, Hempel SJ, Stalnaker SH, Stuart R, Wells L. O-Mannosylation and human disease. *Cell Mol. Life Sci.* doi:10.1007/s00018-012-1193-0 (2012) (Epub ahead of print).

42 Yoshida-Moriguchi T, Yu L, Stalnaker SH *et al.* O-mannosyl phosphorylation of alpha-dystroglycan is required for laminin binding. *Science* 327(5961), 88–92 (2010).

43 Inamori K, Yoshida-Moriguchi T, Hara Y, Anderson ME, Yu L, Campbell KP. Dystroglycan function requires xylosyl- and glucuronyltransferase activities of LARGE. *Science* 335(6064), 93–96 (2012).

44 Cabrera PV, Pang M, Marshall JL *et al.* High throughput screening for compounds that alter muscle cell glycosylation identifies new role for N-glycans in regulating sarcolemmal protein abundance and laminin binding. *J. Biol. Chem.* 287(27), 22759–22770 (2012).

45 Blixt O, Bueti D, Burford B *et al.* Autoantibodies to aberrantly glycosylated MUC1 in early stage breast cancer are associated with a better prognosis. *Breast Cancer Res.* 13(2), R25 (2011).

46 Lu L, Hou X, Shi S, Korner C, Stanley P. Slc35c2 promotes Notch1 fucosylation and is required for optimal Notch signaling in mammalian cells. *J. Biol. Chem.* 285(46), 36245–36254 (2010).

47 Ishikawa HO, Ayukawa T, Nakayama M *et al.* Two pathways for importing GDP-fucose into the endoplasmic reticulum lumen function redundantly in the O-fucosylation of Notch in Drosophila. *J. Biol. Chem.* 285(6), 4122–4129 (2010).

48 Kao YH, Lee GF, Wang Y *et al.* The effect of O-fucosylation on the first EGF-like domain from human blood coagulation factor VII. *Biochemistry* 38(22), 7097–7110 (1999).

49 Valliere-Douglass JF, Brady LJ, Farnsworth C *et al.* O-fucosylation of an antibody light chain: characterization of a modification occurring on an IgG1 molecule. *Glycobiology* 19(2), 144–152 (2009).

50 Liu L, Gomathinayagam S, Hamuro L *et al.* The impact of glycosylation on the pharmacokinetics of a TNFR2:Fc fusion protein expressed in glycoengineered *Pichia pastoris*. *Pharm. Res.* 30(3), 803–812 (2012).

51 Chigira Y, Oka T, Okajima T, Jigami Y. Engineering of a mammalian O-glycosylation pathway in the yeast *Saccharomyces cerevisiae*: production of O-fucosylated epidermal growth factor domains. *Glycobiology* 18(4), 303–314 (2008).

52 Traynor K. Taliglucerase alfa approved for gaucher disease. *Am. J. Health Syst. Pharm.* 69(12), 1009 (2012).

53 Gill DJ, Clausen H, Bard F. Location, location, location: new insights into O-GalNAc protein glycosylation. *Trends Cell Biol.* 21(3), 149–158 (2011).

54 Gill DJ, Chia J, Senewiratne J, Bard F. Regulation of O-glycosylation through Golgi-to-ER relocation of initiation enzymes. *J. Cell Biol.* 189(5), 843–858 (2010).

55 Endo Y, Kobata A. Partial purification and characterization of an endo-alpha-N-acetylgalactosaminidase from the culture of medium of *Diplococcus pneumoniae*. *J. Biochem.* 80(1), 1–8 (1976).

56 Ashida H, Yamamoto K, Murata T, Usui T, Kumagai H. Characterization of endo-alpha-N-acetylgalactosaminidase from *Bacillus sp.* and syntheses of neo-oligosaccharides using its transglycosylation activity. *Arch. Biochem. Biophys.* 373(2), 394–400 (2000).

57 Goda HM, Ushigusa K, Ito H, Okino N, Narimatsu H, Ito M. Molecular cloning, expression, and characterization of a novel endo-alpha-N-acetylgalactosaminidase from *Enterococcus faecalis*. *Biochem. Biophys. Res. Commun.* 375(4), 441–446 (2008).

58 Stadheim TA, Li H, Kett W, Burnina IN, Gerngross TU. Use of high-performance anion exchange chromatography with pulsed amperometric detection for O-glycan determination in yeast. *Nat. Protoc.* 3(6), 1026–1031 (2008).

▪ **HPAEC-PAD application in O-mannose glycan analysis.**

59 Yu G, Zhang Y, Zhang Z, Song L, Wang P, Chai W. Effect and limitation of excess ammonium on the release of O-glycans in reducing forms from glycoproteins under mild alkaline conditions for glycomic and functional analysis. *Anal. Chem.* 82(22), 9534–9542 (2010).

60 Zauner G, Koeleman CA, Deelder AM, Wuhrer M. Mass spectrometric O-glycan analysis after combined O-glycan release by beta-elimination and 1-phenyl-3-methyl-5-pyrazolone labeling. *Biochim. Biophys. Acta* 1820(9), 1420–1428 (2012).

61 Miura Y, Kato K, Takegawa Y *et al.* Glycoblotting-assisted O-glycomics: ammonium carbamate allows for highly efficient o-glycan release from glycoproteins. *Anal. Chem.* 82(24), 10021–10029 (2010).

62 Yamada K, Hyodo S, Matsuno YK *et al.* Rapid and sensitive analysis of mucin-type glycans using an in-line flow glycan-releasing apparatus. *Anal. Biochem.* 371(1), 52–61 (2007).

63 Yamada K, Hyodo S, Kinoshita M, Hayakawa T, Kakehi K. Hyphenated technique for releasing and MALDI MS analysis of O-glycans in mucin-type glycoprotein samples. *Anal. Chem.* 82(17), 7436–7443 (2010).

64 Kozak RP, Royle L, Gardner RA, Fernandes DL, Wuhrer M. Suppression of peeling during the release of O-glycans by hydrazinolysis. *Anal. Biochem.* 423(1), 119–128 (2012).

65 Storr SJ, Royle L, Chapman CJ *et al.* The O-linked glycosylation of secretory/shed MUC1 from an advanced breast cancer patient's serum. *Glycobiology* 18(6), 456–462 (2008).

66 Royle L, Mattu TS, Hart E *et al.* An analytical and structural database provides a strategy for sequencing O-glycans from microgram quantities of glycoproteins. *Anal. Biochem.* 304(1), 70–90 (2002).

▪ **A database for O-glycans separated by different stationary phases.**

67 Marino K, Bones J, Kattla JJ, Rudd PM. A systematic approach to protein glycosylation analysis: a path through the maze. *Nat. Chem. Biol.* 6(10), 713–723 (2010).

68 Jensen PH, Karlsson NG, Kolarich D, Packer NH. Structural analysis of N- and O-glycans released from glycoproteins. *Nat. Protoc.* 7(7), 1299–1310 (2012).

▪▪ **This protocol paper provides experimental details on O-glycan analysis using PGC LC–ESI-MS/MS.**

69 Mittermayr S, Bones J, Doherty M, Guttman A, Rudd PM. Multiplexed analytical glycomics: rapid and confident IgG N-glycan structural elucidation. *J. Proteome Res.* 10(8), 3820–3829 (2011).

70 Melmer M, Stangler T, Premstaller A, Lindner W. Comparison of hydrophilic-interaction, reversed-phase and porous graphitic carbon chromatography for glycan analysis. *J. Chromatogr. A* 1218(1), 118–123 (2011).

71 Staples GO, Bowman MJ, Costello CE *et al.* A chip-based amide-HILIC LC/MS platform for glycosaminoglycan glycomics profiling. *Proteomics* 9(3), 686–695 (2009).

72 Alley WR Jr, Madera M, Mechref Y, Novotny MV. Chip-based reversed-phase liquid chromatography-mass spectrometry of permethylated N-linked glycans: a potential methodology for cancer-biomarker discovery. *Anal. Chem.* 82(12), 5095–5106 (2010).

73 Alley WR Jr, Mechref Y, Novotny MV. Use of activated graphitized carbon chips for liquid chromatography/mass spectrometric and tandem mass spectrometric analysis of tryptic glycopeptides. *Rapid Commun. Mass Spectrom.* 23(4), 495–505 (2009).

74 Bynum MA, Yin H, Felts K, Lee YM, Monell CR, Killeen K. Characterization of IgG N-glycans employing a microfluidic chip that integrates glycan cleavage, sample purification, LC separation, and MS detection. *Anal. Chem.* 81(21), 8818–8825 (2009).

■■ **Reported a fully automated N-glycan analysis workflow using a microfluidic chip LC device couped to ESI-MS.**

75 Ruhaak LR, Deelder AM, Wuhrer M. Oligosaccharide analysis by graphitized carbon liquid chromatography-mass spectrometry. *Anal. Bioanal. Chem.* 394(1), 163–174 (2009).

76 Breloy I. O-glycomics: profiling and structural analysis of mucin-type O-linked glycans. *Methods Mol. Biol.* 842, 165–177 (2012).

77 Knezevic A, Bones J, Kracun SK, Gornik O, Rudd PM, Lauc G. High throughput plasma N-glycome profiling using multiplexed labeling and UPLC with fluorescence detection. *Analyst* 136(22), 4670–4673 (2011).

78 Wada Y, Dell A, Haslam SM *et al.* Comparison of methods for profiling O-glycosylation: human proteome organization human disease glycomics/proteome initiative multi-institutional study of IgA1. *Mol. Cell. Proteomics.* 9(4), 719–727 (2010).

■ **This multicenter study compared different methods for O-glycan analysis.**

79 Robbe C, Michalski JC, Capon C. Structural determination of O-glycans by tandem mass spectrometry. *Methods Mol. Biol.* 347, 109–123 (2006).

80 Xu X, Nagarajan H, Lewis NE *et al.*The genomic sequence of the Chinese hamster ovary (CHO)-K1 cell line. *Nat. Biotechnol.* 29(8), 735–741 (2011).

81 Balaguer E, Demelbauer U, Pelzing M, Sanz-Nebot V, Barbosa J, Neususs C. Glycoform characterization of erythropoietin combining glycan and intact protein analysis by capillary electrophoresis - electrospray - time-of-flight mass spectrometry. *Electrophoresis* 27(13), 2638–2650 (2006).

82 Hanisch FG, Muller S. Analysis of methylated O-glycan alditols by reversed-phase NanoLC coupled CAD-ESI mass spectrometry. *Methods Mol. Biol.* 534, 107–115 (2009).

83 Royle L, Campbell MP, Radcliffe CM *et al.* HPLC-based analysis of serum N-glycans on a 96-well plate platform with dedicated database software. *Anal. Biochem.* 376(1), 1–12 (2008).

84 Doohan RA, Hayes CA, Harhen B, Karlsson NG. Negative ion CID fragmentation of O-linked oligosaccharide aldoses – charge induced and charge remote fragmentation. *J. Am. Soc. Mass Spectrom.* 22(6), 1052–1062 (2011).

85 Guan B, Cole RB. MALDI linear-field reflectron TOF post-source decay analysis of underivatized oligosaccharides: determination of glycosidic linkages and anomeric configurations using anion attachment. *J. Am. Soc. Mass Spectrom.* 19(8), 1119–1131 (2008).

86 Devakumar A, Mechref Y, Kang P, Novotny MV, Reilly JP. Identification of isomeric N-glycan structures by mass spectrometry with 157 nm laser-induced photofragmentation. *J. Am. Soc. Mass Spectrom.* 19(7), 1027–1040 (2008).

87 Zhu F, Lee S, Valentine SJ, Reilly JP, Clemmer DE. Mannose7 glycan isomer characterization by IMS-MS/MS analysis. *J. Am. Soc. Mass Spectrom.* 23(12), 2158–2166 (2012).

88 Gaye MM, Valentine SJ, Hu Y *et al.* Ion mobility-mass spectrometry analysis of serum N-linked glycans from esophageal adenocarcinoma phenotypes. *J. Proteome Res.* 11(12), 6102–6110 (2012).

89 Isailovic D, Plasencia MD, Gaye MM *et al.* Delineating diseases by IMS-MS profiling of serum N-linked glycans. *J. Proteome Res.* 11(2), 576–585 (2012).

90 Zhou W, Hakansson K. Structural characterization of carbohydrates by fourier transform tandem mass spectrometry. *Curr. Proteomics* 8(4), 297–308 (2011).

91 Harvey DJ, Sobott F, Crispin M *et al.* Ion mobility mass spectrometry for extracting spectra of N-glycans directly from incubation mixtures following glycan release: application to glycans from engineered glycoforms of intact, folded HIV gp120. *J. Am. Soc. Mass Spectrom.* 22(3), 568–581 (2011).

92 Mechref Y, Madera M, Novotny MV. Glycoprotein enrichment through lectin affinity techniques. *Methods Mol. Biol.* 424, 373–396 (2008).

93 Tissot B, North SJ, Ceroni A *et al.* Glycoproteomics: past, present and future. *FEBS Lett.* 583(11), 1728–1735 (2009).

94 Zauner G, Kozak RP, Gardner RA, Fernandes DL, Deelder AM, Wuhrer M. Protein O-glycosylation analysis. *Biol. Chem.* 393(8), 687–708 (2012).

95 Dodds ED, Seipert RR, Clowers BH, German JB, Lebrilla CB. Analytical performance of immobilized pronase for glycopeptide footprinting and implications for surpassing reductionist glycoproteomics. *J. Proteome Res.* 8(2), 502–512 (2009).

96 Wuhrer M, Koeleman CA, Hokke CH, Deelder AM. Protein glycosylation analyzed by normal-phase nano-liquid chromatography - mass spectrometry of glycopeptides. *Anal. Chem.* 77(3), 886–894 (2005).

97 Zauner G, Hoffmann M, Rapp E *et al.* Glycoproteomic analysis of human fibrinogen reveals novel regions of O-glycosylation. *J. Proteome Res.* 11(12), 5804–5814 (2012).

98 Nwosu CC, Seipert RR, Strum JS *et al.* Simultaneous and extensive site-specific N- and O-glycosylation analysis in protein mixtures. *J. Proteome Res.* 10(5), 2612–2624 (2011).

99 Zauner G, Deelder AM, Wuhrer M. Recent advances in hydrophilic interaction liquid chromatography (HILIC) for structural glycomics. *Electrophoresis* 32(24), 3456–3466 (2011).

100 Kawasaki N, Itoh S, Yamaguchi T. LC/MSn for glycoprotein analysis: N-linked glycosylation analysis and peptide sequencing of glycopeptides. *Methods Mol. Biol.* 534, 239–248 (2009).

101 Mechref Y. Use of CID/ETD mass spectrometry to analyze glycopeptides. *Curr. Protoc. Protein Sci.* Chapter 12, Unit 12.11, 1–11 (2012).

102 Wuhrer M, Catalina MI, Deelder AM, Hokke CH. Glycoproteomics based on tandem mass spectrometry of glycopeptides. *J. Chromatogr. B Analyt.Technol. Biomed. Life Sci.* 849(1–2), 115–128 (2007).

103 Hanisch FG. O-glycoproteomics: site-specific O-glycoprotein analysis by CID/ETD electrospray ionization tandem mass spectrometry and top-down glycoprotein sequencing by in-source decay MALDI mass spectrometry. *Methods Mol. Biol.* 842, 179–189 (2012).

104 Macek B, Hofsteenge J, Peter-Katalinic J. Direct determination of glycosylation sites in O-fucosylated glycopeptides using nano-electrospray quadrupole time-of-flight mass spectrometry. *Rapid Commun. Mass Spectrom.* 15(10), 771–777 (2001).

105 Nita-Lazar A, Haltiwanger RS. Methods for analysis of O-linked modifications on epidermal growth factor-like and thrombospondin type 1 repeats. *Methods Enzymol.* 417, 93–111 (2006).

106 Harrison R, Hitchen PG, Panico M *et al.* Glycoproteomic characterization of recombinant mouse alpha-dystroglycan. *Glycobiology* 22(5), 662–675 (2012).

107 Lakshminarayanan V, Thompson P, Wolfert MA *et al.* Immune recognition of tumor-associated mucin MUC1 is achieved by a fully synthetic aberrantly glycosylated MUC1 tripartite vaccine. *Proc. Natl Acad. Sci. USA* 109(1), 261–266 (2012).

108 Zhang P, Chan KF, Haryadi R, Bardor M, Song Z. CHO glycosylation mutants as potential host cells to produce therapeutic proteins with enhanced efficacy. *Adv. Biochem. Eng. Biotechnol.* 131, 63–87 (2012).

109 Haryadi R, Zhang P, Chan KF, Song Z. CHO-gmt5, a novel CHO glycosylation mutant for producing afucosylated and asialylated recombinant antibodies. *Bioengineered* 4(2), 90–94 (2012).

110 Zhang P, Haryadi R, Chan KF *et al.* Identification of functional elements of the GDP-fucose transporter SLC35C1 using a novel Chinese hamster ovary mutant. *Glycobiology* 22(7), 897–911 (2012).

111 Steentoft C, Vakhrushev SY, Vester-Christensen MB *et al.* Mining the O-glycoproteome using zinc-finger nuclease-glycoengineered SimpleCell lines. *Nat. Methods* 8(11), 977–982 (2011).

112 Davies SL, Lovelady CS, Grainger RK, Racher AJ, Young RJ, James DC. Functional heterogeneity and heritability in CHO cell populations. *Biotechnol. Bioeng.* 110(1), 260–274 (2013).

» Website

201 NIBRT Database Collection. http://glycobase.nibrt.ie