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Expert Review of Proteomics

ISSN: 1478-9450 (Print) 1744-8387 (Online) Journal homepage: https://www.tandfonline.com/loi/ieru20

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To cite this article: Róisín O'Flaherty, Irena Trbojević-Akmačić, Gordon Greville, Pauline M. Rudd & Gordan Lauc (2018) The sweet spot for biologics: recent advances in characterization of biotherapeutic glycoproteins, Expert Review of Proteomics, 15:1, 13-29, DOI: 10.1080/14789450.2018.1404907

To link to this article: https://doi.org/10.1080/14789450.2018.1404907

Published online: 22 Nov 2017.

EXPERT REVIEW OF PROTEOMICS, 2018
VOL. 15, NO. 1, 13–29
https://doi.org/10.1080/14789450.2018.1404907

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REVIEW

The sweet spot for biologics: recent advances in characterization of biotherapeutic glycoproteins

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ABSTRACT

Introduction: Glycosylation is recognized as a Critical Quality Attribute for therapeutic glycoproteins such as monoclonal antibodies, fusion proteins and therapeutic replacement enzymes. Hence, efficient and quantitative glycan analysis techniques have been increasingly important for their discovery, development and quality control. The aim of this review is to highlight relevant and recent advances in analytical technologies for characterization of biotherapeutic glycoproteins.

Areas covered: The review gives an overview of the glycosylation trends of biotherapeutics approved in 2016 and 2017 by FDA. It describes current and novel analytical technologies for characterization of therapeutic glycoproteins and is explored in the context of released glycan, glycopeptide or intact glycoprotein analysis. Ultra performance liquid chromatography, mass spectrometry and capillary electrophoresis technologies are explored in this context.

Expert commentary: There is a need for the biopharmaceutical industry to incorporate novel state of the art analytical technologies into existing and new therapeutic glycoprotein workflows for safer and more efficient biotherapeutics and for the improvement of future biotherapeutic design. Additionally, at present, there is no 'gold-standard' approach to address all the regulatory requirements and as such this will involve the use of orthogonal glycoanalytical technologies with a view to gain diagnostic information about the therapeutic glycoprotein.

ARTICLE HISTORY
Received 14 June 2017
Accepted 10 November 2017

KEYWORDS
Capillary electrophoresis (CE); critical quality attribute (CQA); electrospray ionization-mass spectrometry (ESI-MS); fusion protein; glycopeptide; glycoprotein; immunoglobulin (IgG); monoclonal antibody (mAb); MS/MS; ultra performance liquid chromatography (UPLC)

1. Introduction

Glycosylation of biotherapeutic glycoproteins plays an important role in cellular communication *in vivo* and can alter function, safety, and efficacy of the drug. It is estimated that more than 50% of human proteins are glycosylated and approximately 90% of these contain *N*-linked glycans or a combination of *N*-linked and *O*-linked glycans [1]. Many of the protein-based biotherapeutics approved or in clinical trials are glycoproteins, including the large class of monoclonal antibodies (mAbs), cytokines and enzyme replacement therapies. mAbs based on immunoglobulin G 1 (IgG1), an antibody for Fc receptor binding are frequently exploited as therapeutic agents, see Figure 1. Human IgG1 antibodies contain two conserved *N*-glycan sites on the Fc (fragment crystallizable) at Asn 297 on each heavy chain, and up to four additional *N*-glycan sites on the Fab (fragment antibody binding) [2]. *N*-glycans contain a common chitobiose core of two *N*-acetylglucosamine residues (GlcNAc) linked to an asparagine residue on the glycoprotein via an amide bond and extended with a trimannosyl core, see Figure 1. This core structure may be decorated with additional monosaccharide residues such as mannose (Man), GlcNAc, galactose (Gal), fucose (Fuc), bisecting GlcNAc, and sialic acids such as *N*-acetyl neuraminic acid (NANA) or *N*-glycolyl neuraminic acid (NGNA). Biosynthesis of eukaryotic *N*- and *O*-glycosylation has been extensively described in the literature previously [4,5].

Recombinant cytokines are used in a wide variety of infectious and autoimmune diseases, in immunocompromised patients with AIDS (acquired immune deficiency syndrome), and in neoplasia [6]. Recombinant human interferon-β 1a glycoproteins such as Avonex and Rebif are widely used as a first-line treatment for multiple sclerosis (MS) [7]. The native glycoprotein contains a single *N*-glycan site and the recombinant versions produced in Chinese Hamster Ovary (CHO) cells, mouse epithelial cells, and human lung adenocarcinoma cells all contain the most abundant native biantennary complex-type *N*-glycans present in the native glycoprotein [8]. Recently, glycoengineered variants have been produced using site-specific hyperglycosylation via site-directed mutagenesis to improve pharmacokinetic properties of the glycoprotein [7]. Therapeutic glycoproteins have also found applications in enzyme replacement therapies such as agalsidase alfa (Replagal) and agalsidase beta (Fabrazyme) which have changed the treatment of Anderson-Fabry disease. The recombinant enzyme agalsidase alfa is produced in a cultured human cell line whereas the agalsidase beta is produced in CHO cells, both glycoproteins contain complex type *N*-glycans [9,10].

In contrast to *N*-glycans, *O*-glycans on therapeutic glycoproteins do not contain a common core structure [11]. They are most commonly attached to serine/threonine residues on protein/peptide structures but can also be attached via hydroxylysine, hydroxyproline, or tyrosine residues. Mucin type *O*-glycans, which do contain common core structures, are noteworthy and are found

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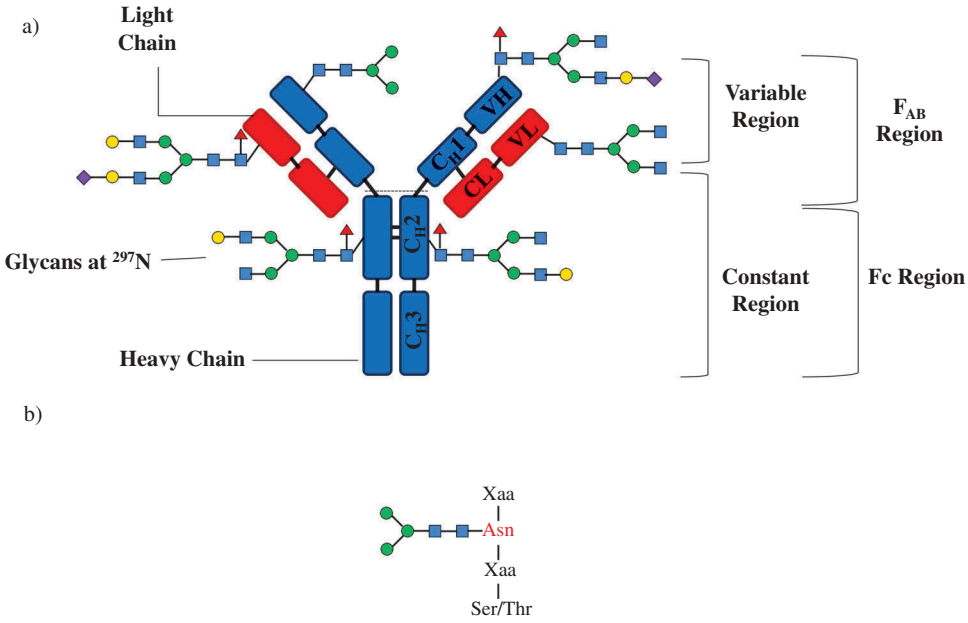
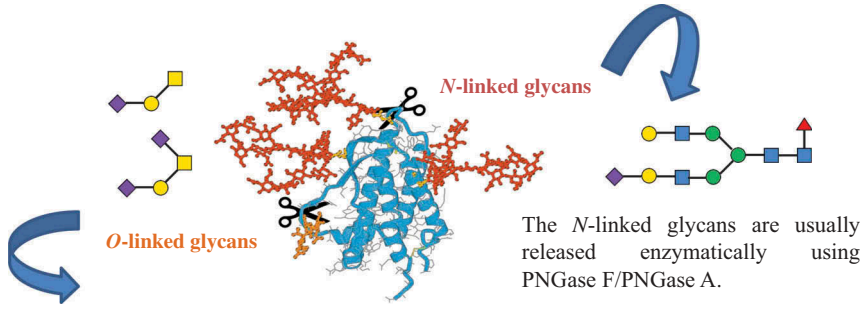


Figure 1. IgG antibody structures and *N*-glycan structures. a) Schematic representation of the glycoprotein IgG, a common motif for mAbs. Glycosylation sites are decorated with *N*-glycans: two conserved fragment crystallisable (Fc) *N*-glycan sites linked to asparagine 297 in C_H2 domains and four potential fragment antibody binding (Fab) *N*-glycan sites linked to the variable regions on the heavy and light chains. Depending on the glycoform, the *N*-glycans may be attached to the variable regions of the light chain, the heavy chain or both and asymmetric Fc and Fab *N*-glycans can also exist [2], although many mAbs contain only Fc glycosylation. **b)** All *N*-glycans contain common core pentasaccharide ($Man_3GlcNAc_2$) containing a chitobiose core of two *N*-acetylglucosamine (GlcNAc) residues attached to asparagine residue on protein backbone via an amide bond. Three mannose (Man) residues are attached to the outer GlcNAc residue. The Man residues can be extended with an array of additional glycans such as GlcNAc, galactose and sialic acids. The glycan structures are depicted with SNFG nomenclature [3].



	***N*-glycans**	***O*-glycans**				
		Ser	Thr	Hyp	Tyr	Ser /Thr (COOH)
PNGaseF/PNGase A	Y	N	N	N	N	N
Hydrazinolysis	Y	Y	Y	U	U	U
β-elimination	N	Y	Y	N	N	N

Figure 2. Methods of release of *N*- and *O*-linked glycans for biotherapeutics shown on erythropoietin (EPO) as a representative example. *N*- and O-glycan structures on the glycoprotein are presented in red and orange respectively and can be cleaved at the site of attachment to the protein. *N*-linked glycans are usually cleaved enzymatically using PNGase F or PNGase A, whereas *O*-linked glycans are more commonly cleaved using chemical means, with hydrazinolysis and β-elimination methods. The *O*-glycans are attached to the glycoproteins via amino acid residues serine (Ser), threonine (Thr), hydroxyproline (Hyp), tryrosine (Tyr) on the side chains but can also be located on carboxylic moieties of either serine/threonine. The table inset presents which type of glycans can be cleaved using the specified methods, Y for yes, N for no and U for uncertain. (Permission for reprint of EPO structure granted by Dr. Mark Wormald, Oxford Glycobiology Institute).

on mucins as well as many therapeutic glycoproteins, see representative *O*-glycan structures in Figure 2 [12,13]. The initiation step in synthesis is the attachment of the first carbohydrate residue, GalNAc, to selected serine and threonine residues in proteins using GalNAc-transferases. This transformation is harnessed in GlycoPEGylation. GlycoPEGylation of recombinant therapeutic

proteins is employed to enhance pharmacodynamic properties of the biologic whereby polyethylene glycol is transferred to the recombinant protein expressed in *Escherichia coli* (lacking mucin type *O*-glycosylation) to specific acceptor sites directed by GalNAc-transferases [13–15].

By characterization of released *N*- and *O*-glycan structures, glycopeptide, and intact glycoprotein structures in biotherapeutics, we can identify their origin (mammalian, plant, or insect) and mitigate undesired immunological responses using glycoengineering approaches including systems such as CRISPR-Cas9 [16,17]. This review gives an overview of biotherapeutics recently approved in 2016 and 2017 with consideration toward suitable glycoanalytical technologies. It also showcases analytic methodologies for the characterization of therapeutic glycoproteins with a focus on released *N*- and *O*-glycan characterization, glycopeptide characterization, and glycoprotein characterization, see Figure 3. Additional reviews relating to glycoprotein characterization are included [18–21].

2. Glycoprotein biotherapeutics approved by FDA in 2016 and 2017 and considerations for glycoanalytics

Glycosylation is a CQA that must be presented to regulatory bodies for approval of therapeutic glycoproteins. In the past decade, the emergence of mAbs has dominated the global biopharmaceutical market [22]. At the same time, state-of-the-art glycoanalytical technologies have emerged to provide detailed process and product information. To gain insight into the analytical techniques that need further development, we first need to understand the shape of the current biopharmaceutical landscape. This section therefore seeks to showcase the degree of recently approved biopharmaceuticals that bear glycosylation in which analytical technologies will be crucial for the regulatory process.

To date in 2017 and 2016, 11 and 14 new Biological License Applications (BLAs) were granted respectively for *in vivo* therapeutics from the FDA's Centre for Drug Evaluation and Research (CDER), see Table 1 [23]. The BLA licenses granted in 2017 and 2016 for *in vitro* therapeutics are not discussed in this section as they are outside the scope of this review. Of the therapeutics approved in 2016 and 2017, 24 drugs (96%) contain glycosylation, of which analytical techniques such as ultra performance liquid chromatography (UPLC), mass spectrometry, or capillary electrophoresis (CE) remain vital tools for validation and quality control, discussed in Sections 3–5. Perhaps not surprisingly, only one BLA license granted by the CDER in 2016 and 2017 does not contain glycosylation, Atezolizumab which interestingly was designed for reduced Fc effector function by eliminating glycosylation binding. Twenty one (84%) of the therapies are based on mAbs, two therapies (8%) as antibody drug conjugates (ADCs), one therapy (4%) as a fusion protein product, and one therapy as enzyme

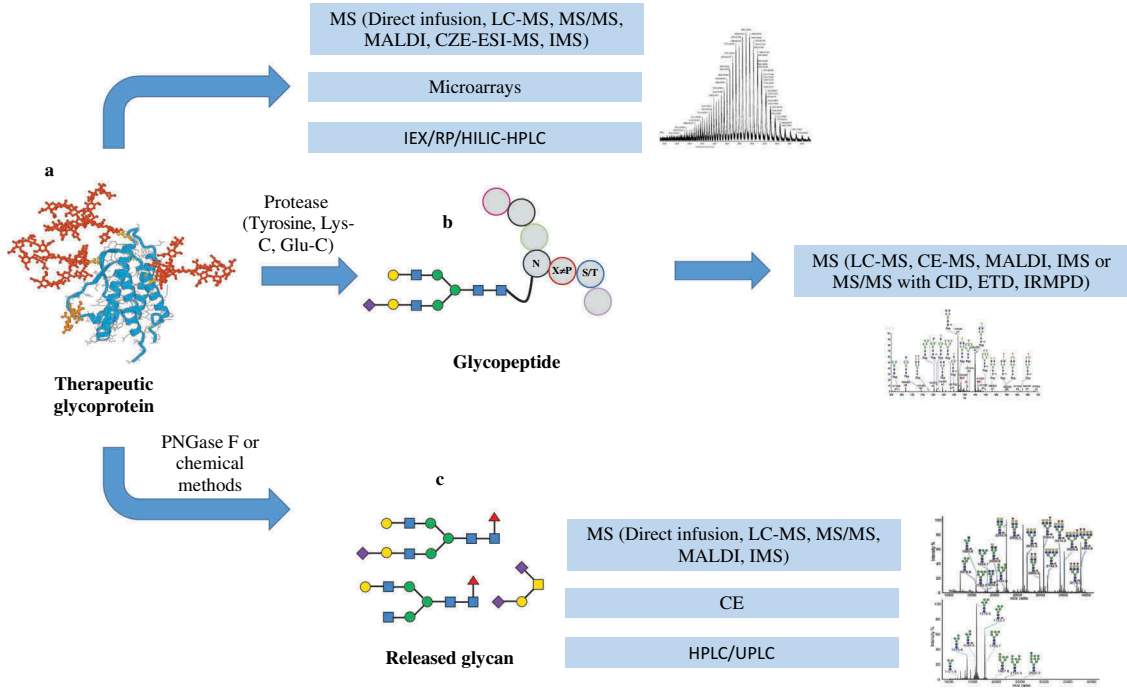


Figure 3. Schematic for general analytical strategies for therapeutic glycoprotein characterization. A) Intact therapeutic glycoprotein characterization can be conducted using mass spectrometry (MS) techniques such as direct infusion, liquid chromatography-mass spectrometry (LC-MS), tandem mass spectrometry (MS-MS), matrix assisted laser desorption ionization (MALDI), capillary zone electrophoresis (CZE)-MS or ion mobility spectroscopy (IMS). Alternatively microarrays can be employed using commercial or custom lectin/glycan binding protein GBP chips. Other general strategies employ chromatography techniques such as ion exchange chromatography (IEX), reverse-phase (RP) chromatography or hydrophilic (HILIC) chromatography are these technologies are usually implemented orthogonally with other techniques. B) Glycopeptide analysis is conducted with the initial treatment of therapeutic glycoprotein with Protease such as trypsin, Lys-C or Glu-C to release glycopeptide fragments. This technique offers the advantage of site specific glycosylation information and glycopeptides are characterized using mass spectrometry techniques such as LC-MS, CE-MS, MALDI, IMS or MS/MS using collision induced dissociation (CID), electron transfer dissociation (ETD) or infrared multiphoton dissociation (IRMPD) for fragmentation. C) Released glycan analysis characterisation for therapeutic glycoproteins is performed using either enzymatic release for *N*-glycans or chemical release for *O*-glycans. Analytical methodologies include MS techniques such as direct infusion, LC-MS, MS/MS, MALDI, IMS. Alternative analytical methods include CE or (Ultra)-high performance liquid chromatography (HPLC/UPLC). The glycan structures are depicted with SNFG nomenclature [3]. (Permission for reprint of EPO structure granted by Dr. Mark Wormald, Oxford Glycobiology Institute).

Table 1. Summary of approved in vivo therapeutic biologics under Biologics License Applications (BLAs) for 2016 and 2017 (until 30 September 2017) from FDA's Centre for Drug Evaluation and Research (CDER). Approvals by the Center for Biologics Evaluation and Research (CBER) are not included in this table. Columns include, BLA numbers, drug name provided as trademark name, the active ingredient, glycosylation status (glycans) as Y or N for yes or no, biosimilar status as B for biosimilar and the tradename of the innovator drug in brackets and the last column is the description. List of all CDER approved drugs can be found in the FDA Purple Book [23].

Year	Drug name (BLA number)	Active ingredient	Glycosylation	Biosimilar	Description
2016	Zinplava (761046)	Bezlotoxumab	Y		Recombinant human IgG1 for *Clostridium difficile* toxin B binding
	Lartruvo (761038)	Olaratumab	Y		Recombinant human IgG1 for human PDGFR-α binding
	Stelara (761044)	Ustekinumab	Y		Recombinant human IgG1κ for IL-12 and IL-23 binding
	Zinbryta (761029)	Daclizumab	Y		Recombinant humanized IgG1κ for CD25 binding
	Tecentriq (761034)	Atezolizumab	N		Recombinant Fc-engineered IgG1κ for PD-L1 binding
	Cinqair (761033)	Reslizumab	Y		Recombinant humanized IgG4κ for IL-5 binding
	Taltz (125521)	Ixekizumab	Y		Recombinant IgG4 for IL-17A binding
	Anthim (125509)	Obiltoxaximab	Y		Recombinant chimeric IgG1κ for *Bacillus anthracis* toxin binding
	Amjevita (761024)	Adalimumab-atto	Y	B (Humira)	Recombinant human IgG1 for TNF binding
	Erelzi (761042)	Etanercept-szzs	Y	B (Enbrel)	Recombinant dimeric fusion protein with portion of the human TNFR linked to a modified human IgG1 Fc
	Inflectra (125544)	Infliximab-dyyb	Y	B (Remicade)	Recombinant chimeric IgG1κ for human TNFα binding
2017	Bavencio (761049)	Avelumab	Y		Recombinant human IgG1λ for PD-L1 binding
	Ocrevus (761053)	Ocrelizumab	Y		Recombinant humanized IgG1 for CD20 binding
	Dupixent (761055)	Dupilumab	Y		Recombinant human IgG4 for IL-4Rα binding
	Brineura (761052)	Cerliponase alfa	Y		Recombinant human rhTPP1, a lysosomal exopeptidase for cleavage of *N*-terminal tripeptides
	Imfinzi (761069)	Durvalumab	Y		Recombinant human IgG1κ for PD-L1 binding
	Tremfya (761061)	Guselkumab	Y		Recombinant human IgG1λ for IL-23 blocking
	Besponsa (761040)	Inotuzumab ozogamicin	Y		Antibody drug conjugate with recombinant humanized IgG4κ for CD22 binding, N-acetyl-gamma-calicheamicin for cytotoxicity and an acid-cleavable linker
	Rituxan Hycela (761064)	Rituximab and hyaluronidase human	Y		A combination of rituximab and hyaluronidase human. Rituximab is a recombinant chimeric murine/human IgG1κ against the CD20 antigen. Recombinant human hyaluronidase is an endoglycosidase used to increase the dispersion and absorption of coadministered drugs
	Kevzara (761037)	Sarilumab	Y		Recombinant human IgG1 for IL-6 binding
	Mylotarg (761060)	Gemtuzumab ozogamicin	Y		Antibody-drug conjugate with recombinant humanized IgG4κ for CD33 binding that is covalently linked to the cytotoxic agent N-acetyl gamma calicheamicin
	Benlysta (761043)	Belimumab	Y		Recombinant human IgG1λ specific for soluble human B 327 lymphocyte stimulator protein
	Cyltezo (761058)	Adalimumab-adbm	Y	B (Humira)	Recombinant human IgG1g for human TNF binding
	Mvasi (761028)	Bevacizumab-awwb	Y	B (Avastin)	Recombinant humanized I IgG1 for VEGF binding
	Renflexis (761054)	Infliximab-abda	Y	B (Remicade)	Recombinant chimeric IgG1κ for human TNFα binding

replacement therapy (4%), see Table 1. Of the mAb products (84%) all are expressed as recombinant proteins, of which 18 are IgG1 derivatives and 3 are IgG4 variants. One notable addition in this category is the combination of mAb with an enzyme to speed up adsorption, reducing the drug administration time for the patient (Rituxan Hycela). However, this advance is unlikely to change the face of glycoanalytics unless the enzyme affects the therapeutic glycosylation process. On the other hand, the small shift toward ADCs and fusion proteins in recent years will add another level of complexity for glycoanalytical testing. For instance, drug antibody ratio (DAR) of ADCs is important for therapeutic efficacy and pharmacokinetics, therefore control of DAR in synthesis processes requires advances for fast real-time monitoring for quality control [24].

Many of these recently approved biotherapeutics are expressed in CHO or Murine Myeloma cell lines (NS0 and SP2/0). Protein production in these mammalian expression systems has the advantage that they contain 'human-like' mammalian type glycans for therapeutic use compared to bacterial, plant, or insect expression systems. Depending on the expression systems, different glycoforms are produced and can alter the pharmacokinetics, efficiency, and targeting of the glycoprotein [25]. CHO cells are the most commonly employed expression system in the biopharmaceutical industry and are desirable for many reasons including the high yields (2–6 g/L) for antibody products. On the other hand, CHO cells contain both immunogenic galactose-α1,3-galactose (α-Gal) epitopes [26,27], contrary to the assumption that CHO lacks the biosynthetic machinery [28,29], and NGNA glycans not indigenous to humans [30] and as such the need to identify, characterize, and minimize these glycans is paramount. The murine cell lines NS0 and Sp2/0 are also used for recombinant mAb production despite expressing the two predominant immunogenic glycan epitopes for humans as observed for CHO cells, α-Gal, and NGNA glycans [29]. Therefore, careful consideration is needed with regards to their glycoanalytical technologies for characterization of the CQAs

with a strong focus on characterization of known antigenic or immunogenic glycan epitopes.

Since approval of the first biosimilar in 2006 in Europe, there is an increasing trend toward biosimilar production in an effort to lower patient costs, especially in China and India, with both countries expected to grow their biopharmaceutical industries at the fastest compound annual growth rate of 30% and 29% from 2013 to 2018 [31]. Detecting differences in glycosylation between innovator drugs and biosimilars can be crucially important to ensure the safety and efficacy of these emerging drugs and the criteria is met by fulfilling the requirements of the International Council for Harmonization's (ICH's) Q6B. Crucially, the presence/absence of even one sugar residue can alter the biologic activity of the agent [32]. Biosimilar approvals by FDA in 2016 include Inflectra (Infliximab), Erelzi (Etanercept), and Amjevita (Adalimumab), see Table 1 [23,33]. More recently in 2017 to date three biosimilars have also been approved by the CDER including Cyltezo (Adalimumab), Mvasi (Bevacizumab), and Renflexis (Infliximab). The comparison of the glycan profiles of the biosimilar compared to the innovator drug is one of the regulatory requirements, whereby the biosimilars are required to be similar in structure to their biologic reference product but are neither expected nor required to contain identical active substances [34,35]. Taking the changing climate for modern biopharmaceuticals into account glycoanalytical advances are discussed in Sections 3–5.

3. Released *N*- and *O*-glycan analysis of therapeutic glycoproteins

Glycosylation analysis of therapeutic proteins on the level of released *N*-, or *O*-glycans gives a snapshot of glycan levels from the pool of glycoprotein molecules. Profiling of *N*- and *O*-linked glycans is an extremely important part of the quality control strategy for ensuring lot and batch consistency for therapeutic glycoproteins. Glycoprofiles may vary greatly depending on the source of the therapeutic glycoprotein and certain glycan structures may cause anything from decreased serum half-life of a compound to an extreme immune response [36,37]. For this reason, WHO guidelines on therapeutic proteins and the International Conference on Harmonization (ICH) Q6B mandate state that posttranslational modifications such as glycosylation should be identified and accurately characterized [38,39]. Glycosylation is also sensitive to alterations in the host cells environment. Because of this, regular glycoprofiling is necessary to ensure batch to batch consistency. Finally because of the phenomena known as the 'patent cliff', there are a large number of biosimilars entering the market. Both the European Medicines Agency and the FDA require a detailed comparison of the sugar structures through glycan profiling as part of the process in confirming biosimilarity [39].

Released glycan analysis is very convenient for fast screening of therapeutic glycoproteins and QC assays. However, on the other hand, it does not provide information relating to glycosylation on each potential glycosylation site of a therapeutic glycoprotein. Released *N*- or *O*-glycoprofiling is based on several major steps – purification of therapeutic glycoprotein from cell culture, release of glycans from purified glycoprotein, chemical modification of released glycans for the subsequent detection (usually with fluorescent detection), clean-up procedure to remove excess reagents, and finally detection of individual glycan species usually after chromatographic or gel electrophoresis separation.

3.1. Purification of therapeutic glycoproteins

Protein purification contributes to a large portion of the expense accrued in therapeutic protein production [40]. For this reason, the design of a simple, cost-effective, protein isolation, and purification process is key to developing a production strategy. Currently, the three main methods for therapeutic glycoprotein purification are affinity chromatography, ion exchange chromatography, and fusion tags [41–43]. Affinity chromatography is most commonly exploited in the case of therapeutic glycoprotein purification of a single protein (e.g. a mAb). The technique affords separation of biomolecules based on antibody-antigen/enzyme-substrate/receptor-ligand binding and is generally a useful method for targeting a single protein. Protein A is a good example of this and is commonly employed in the biopharmaceutical industry [44,45]. Protein A is found in the cell wall of the bacteria *Staphylococcus aureus* and was found to strongly bind IgG antibodies which are exploited for the capture of therapeutic mAbs. However, the production of antibody purification systems can be expensive so other intrinsic physicochemical properties of the protein can be exploited such as the net charge of the glycoprotein through ion exchange chromatography. This method is employed for the purification of recombinant human erythropoietin (rhEPO) [46]. As there may be other proteins with a similar charge in the media, ion exchange chromatography is usually performed in combination with other separation techniques such as HPLC and size exclusion chromatography. Again, these additional steps add time and cost to the purification process. Some proteins do not have any unique characteristics, for this reason an affinity tag, such as glutathione-S-transferase or a histidine tag (HisTag) can be added as a DNA sequence to the gene of interest [47]. The gene product is now known as a recombinant fusion protein. Additional tags which confer other desirable properties to a recombinant glycoprotein, such as increased solubility, can also be added [40]. While the tags give great convenience in the purification step, they may also be immunogenic once they come into contact with the recipient's immune system. Therefore, the tag must be removed, usually with an endoprotease [47] and depending on the design of the fusion protein the complete removal of the tag is not always possible. This must be taken into consideration when designing the purification method.

The high costs associated with purification of expressed proteins in downstream processing is recognized as a major challenge for biopharmaceutical industry [48] and has encouraged creativity in the field to investigate cost-effective solutions. Improvements including alternatives to Protein A chromatography [49], investigations into Protein A fouling [50] and incorporation of filtration methods [45] prior to Protein A chromatography have all been explored in this context. It is clear, however, that more efforts are needed for their mainstream application in the biopharmaceutical industry.

3.2. Methods for N-glycan release from therapeutic glycoproteins

Analysis on the level of released glycans allows screening of therapeutic glycoproteins for any variations in glycan structure, which can alter efficacy, half-life, or immunogenicity of the therapeutic. Glycoproteins from different batches have to have consistent released glycan profiles in order to meet regulatory requirements or pass quality control. Generally, there are two approaches for *N*-glycan release – chemical deglycosylation (hydrazinolysis) and enzymatic reaction.

Hydrazinolysis is based on glycans release from glycoprotein using anhydrous hydrazine [19,51] and it requires clean initial sample and several steps to minimize potential side reactions. In addition to being more labor intensive, efficiency of deglycosylation is usually lower and loss of glycans through degradation in the procedure are higher compared to enzymatic deglycosylation, making it less suitable for biopharmaceutical use. On the other hand, glycosidases such as *N*-glycosidase F (PNGase F) are very selective and specific, and can be added directly to a denatured glycoprotein solution, that is, for recombinant erythropoietin biotherapeutics [52]. Alternatively, the glycosidases can be employed in the form of immobilized enzyme reactors. Many examples in the literature [53–55] describe the release of *N*-glycans from glycoprotein standards such as IgG and fetuin using this methodology and it can be harnessed in future applications for method development of biopharmaceuticals to minimize associated enzyme costs. *N*-glycan release is performed overnight, in a few hours or even a few minutes depending on the conditions and experimental setup for the recombinant glycoprotein [56] and novel approaches such as microwave-assisted or pressure cycling technology [57,58] can also be applied to biopharmaceutical glycoanalytical platforms in the future.

PNGase F is most commonly used for profiling of total *N*-glycans in biopharmaceuticals, except if they have α1,3 core-fucose residues, that is, if a therapeutic glycoprotein has been expressed in insect or plant cells. Glycans containing α1,3 linked fucose on the reducing-terminal GlcNAc on the chitobiose core may instead be released using *N*-glycosidase A (PNGase A; also known as glycoamidase A, see Figure 2) [19,59], while endoglycosidase H (Endo H) is very convenient for specific analysis of high-mannose glycans [60], useful for characterization at the development stage for biotherapeutics such as yeast or CHO produced therapeutic glycoproteins.

In the recent few years, several customized enzymes have been developed for characterization of IgG antibodies by the company Genovis and are very useful for *N*-glycan characterization of antibody-based therapeutics. For example, IdeS (commercially known as FabRICATOR by Genovis, and also available from Promega) specifically digests human IgG below the hinge region and results in an $F(ab')_2$ fragment and two Fc fragments, therefore enabling characterization of *N*-glycans from both Fab and Fc fragments after their separation [61–63]. Another application of IdeS is separate characterization of glycans from Fc part and fusion part of Fc fusion proteins (e.g. abatacept) that have noncanonical hinge region [64]. Similarly, IdeZ protease has the same specificity as IdeS, but with improved activity toward mouse IgG2a and IgG3 subclasses, and has also been used for characterization of Fc fusion protein (human cell line-derived chimeric human Coagulation Factor VIII-Fc) [65]. Moreover, IgGZERO (EndoS) specifically cleaves after the first GlcNAc residue in the Fc-region of native IgG, while GingisKHAN digests only human IgG1 between K223 and T224 producing a homogenous pool of Fab and Fc fragments [66]. Although, these enzymes are still a higher price, the advantage over widely used PNGase F is the possibility of targeted *N*-glycan analysis, which is especially useful since different regions/parts of Fc fusion proteins can have distinct glycosylation profiles and functions. This will find application in development programs of therapeutic glycoproteins.

Purified therapeutic glycoproteins are generally denatured and disulfide bonds reduced and alkylated prior to enzymatic glycan release to minimize steric hindrance and make glycans more accessible to an enzyme. For example, erythropoietin is known to be resistant to PNGase F digestion without prior denaturation [52]. However, improved recombinant glycosidases are being developed that allow deglycosylation of native glycoproteins, for example, IgGZERO (EndoS) and GlycINATOR (EndoS2) from Genovis. Another trend is development of rapid recombinant versions of PNGase F (for example Rapid PNGase F by New England Biolabs), which deglycosylate the glycoprotein substrate in only few minutes, therefore significantly speeding up the analysis of therapeutic glycoproteins during QC, as well as enabling fast screening of a therapeutic glycoprotein during the production process.

Exoglycosidase digestions are often used orthogonally with *N*-glycan release strategies for characterization of therapeutics at developmental stages, their relative high costs hinder their routine use in QC assays. Glycoprotein digestion with neuraminidase and subsequent UPLC or MS analysis could provide information about presence and amount of sialic acids [64,65], and other exoglycosidase digestions are used for characterization of other glycan moieties [67,68]. Additionally, level of sialic acids (Neu5Ac, Neu5Gc, and *O*-acetylated sialic acids) could be determined by mild hydrolysis and subsequent labeling with 1,2-diamino-4,5-methylenedioxybenzene using a kit developed by Ludger Ltd, which has recently been used in analysis of three epoetin alpha products [69].

3.3. Methods for O-glycan release from therapeutic glycoproteins

Unlike *N*-glycans, *O*-glycan release is challenging due to lack of universal enzymes to cleave the *O*-glycans from proteins. Thus chemical treatments have been employed to release *O*-glycans from biopharmaceuticals such as reductive β-elimination methods in dimeric fusion protein Etanercept [70,71] and recombinant erythropoietins [12,72] or hydrazinolysis, see Figure 2 [73,74]. Reductive β-elimination is useful for releasing *O*-glycans attached to serine or threonine amino acid residues. The glycoprotein is treated with base (usually mild sodium hydroxide) to release the peptide/protein fraction as a dehydroamino acid derivative and the glycan portion is converted into an alditol by reduction (usually using sodium borohydride solution) to ensure minimal conversion into undesired

'peeling' products obtained by degradation of the glycans. However the reductive β-elimination reaction is limited as it does not cleave *O*-glycans on amino acid residues hydroxylysine, hydroxyproline, tyrosine, or certain serine/threonine residues (whereby glycans are decorated on the carboxylic acids). The conversion to the alditol also prevents the reductive amination needed for the attachment of a fluorophore or chromophore [75]. Matrix-assisted laser desorption/ionization–time of flight (MALDI-TOF) mass spectrometry serves as a suitable technique to characterize the *O*-glycans using this method as the glycans can be permethylated to increase the ionization potential of the glycans [76].

Hydrazinolysis serves as an alternative method for *O*-glycan detection but can be an arduous method that involves prepurification of the glycoprotein, anhydrous hydrazine, elevated temperatures, removal of excess hydrazine, and re-acetylation of *N*-acetyl functionalities [73]. Commercial kits are available including GlycoProfile™ β-Elimination Kit (Sigma Aldrich) and GlycoRelease™ Glycan Hydrazinolysis Kit (Prozyme) which may be useful for preliminary structural studies for therapeutic glycoproteins. However the challenges associated with preparation of released *O*-glycans using this method limits its use for therapeutic glycoproteins in QC assays.

Combinations of enzymes for *O*-glycan characterization, although currently limited, may also be useful for glycosylated biopharmaceuticals, including the use of *O*-glycanase (available from Prozyme) which cleaves unsubstituted Ser/Thr linked Gal-GalNAc from proteins. The exoglycosidase can be used in conjunction with other enzymes/cocktails of enzymes such as prO-LINK Extender™ Kit for Complex *O*-Linked Glycans (Prozyme) or Protein Deglycosylation Kit (New England Biolabs) as well as other commercial enzymes to decipher the *O*-glycan monosaccharides. Some examples in the literature have highlighted enzymatic approaches to this end [77]; however, their main application at present remains as useful tools to discover if proteins are glycosylated in SDS page gels. Ongoing efforts in the field to develop a universal *O*-glycanase, synonymous with PNGase F for *N*-glycan characterization, if successful, may dramatically change the face of *O*-glycan characterization of therapeutic glycoproteins in the future.

3.4. Fluorescent labeling of N-glycans and O-glycans

Glycans are not fluorophores and for their detection and quantification by UPLC or CGE-LIF a labeling reaction with fluorescent tag is necessary step in the workflow. However depending on the release method for *O*-glycans, reductive amination, Michael addition, and hydrazide labeling may not be suitable as they all require the reducing end of the glycan, which is not present on *O*-glycans released by reductive β-elimination. On the other hand, *O*-glycans released by other methods may be fluorescently labeled. Several fluorescent labels have been described for glycan labeling for glycoproteins: 2-aminopyridine (PA) [78], 2-aminobenzoic acid (2-AA) [79,80], 2-aminobenzamide (2-AB) [56,81], procainamide (4-amino-*N*-(2-diethylaminoethyl) benzamide) [69,82] and more recently aminoquinoline carbamate (AQC) [44] and RapiFluor-MS (Waters) for the UPLC and MS techniques. For CGE-LIF, 8-aminopyrene-1,3,6-trisulfonate (APTS) and 2-amino-1-naphthalenesulfonic acid (2-ANSA) can be used. However, routine use of some of these reagents in the biopharmaceutical industry is limited due to strict requirements for QC assays (i.e. reagents must be purchased from reputable commercial sources). Traditionally commercial kits such as LudgerTag 2-AB Labeling Kit (Ludger Ltd), 2-AB Labeling Kit (QA-Bio), GlycoWorks 2-AB Labeling Reagent Kit (Waters), Glycoprofile 2-AB Labeling Kit (Sigma-Aldrich), and GlykoPrep Kit (Prozyme) are employed for 2-AB labeling, LudgerTag 2-AA Labeling Kit (Ludger Ltd) and Glycoprofile 2-AA Labeling Kit (Sigma-Aldrich) for 2-AA labeling or the most recently released fast release kits such as GlycoWorks RapiFluor-MS *N*-glycan kit (Waters) or Fast Glycan Labeling and Analysis Kit (SCIEX) can be employed.

Geyer and Geyer have reported PA as one of the most commonly used label for the chromatographic glycan analysis [19]. However, since commercially available PA is not sufficiently pure for this application, it has a limited use due to the necessary additional step of recrystallization. 2-AA label is negatively charged and due to this property widely used for labeled glycans detection by techniques where separation and detection are based on charge, for example, in CGE [83,84], positive-mode MALDI-TOF-MS [79,85], and in negative-mode MALDI-TOF-MS [86,87], allowing the detection of both neutral and sialylated glycans in the same experiment. For example, 2-AA labeling has been employed for comparison of a biosimilar to an innovator biologic [88]. 2-AB is a neutral nonselective label that allows accurate quantitative measurement of relative amounts of individual glycans, both neutral and charged ones. It is compatible with most chromatographic and mass spectrometric methods used for glycan separation and analysis, and is mostly used for glycan profiling by LC. On the other hand, however, 2-AB has a poor ionization efficiency when glycan structures are analyzed by ESI–MS [89]. Comparison of 2-AA and 2-AB showed that 2-AA labeled glycans have higher intensities than 2-AB labeled glycans when analyzed by RP-LC-MS analysis [90] or chromatographic analysis [91]. With a shift toward the more commonplace use of mass spectrometry in biopharmaceutical characterization 2-AA labeling may be employed more frequently in the future.

The trend in the last few years is to develop labeling reagents that allow efficient and sensitive detection, identification, and quantification of labeled glycans both by chromatographic and MS techniques in the scientific community. Procainamide ((4-amino-N-(2-diethylaminoethyl) benzamide) is an aromatic amine tag that allows glycan profiling and identification both by UPLC-FLR and ESI-QTOF-MS [82], since it provides efficient ionization due to the amine moiety. Procainamide labeling allows the glycan analysis on multiple levels using the same sample, which significantly reduces the amount of initial sample that is needed for the glycoprotein characterization and time needed for sample preparation. Comparison of 2-AB and procainamide labeled glycans showed higher fluorescent intensities of glycans labeled with procainamide than with 2-AB, allowing more sensitive quantification of less abundant glycans [92]. In addition to that, procainamide labeled glycans have excellent chromatographic peak resolution when analyzed by UPLC-FLR and up to 10–50 fold more efficient ionization than 2-AB labeled glycans when analyzed by ESI–MS [92]. Although it still has not been routinely used for glycan analysis in biopharmaceuticals, due to the number of advantages and commercial availability of

procainamide kits (LudgerTag PROC glycan labeling kit, Ludger Ltd, and InstantPC, Prozyme), this may change in the future.

Another labeling reagent that allows high sensitivity detection by both fluorescence and MS (positive ion mode ESI-MS) is RapiFluor-MS (Waters Corporation) and is suitable for therapeutic glycoprotein characterization. This label has an *N*-hydroxysuccinimide carbamate reactive group, a quinoline fluorophore, and a basic tertiary amine [93]. Additional advancement is the possibility of labeling reaction at room temperature within only few minutes, compared to the traditional labeling reagents which require sample incubation at 65°C for 2–3 h [44,93]. Two other currently available 'instant labeling' solutions in addition to RapiFluor-MS are InstantAB from Prozyme and 6-aminoquinoyl-*N*-hydroxysuccinimidyl carbamate (AQC) commercially available by Synchem and Waters Corporation, but can also be synthesized in-house [44,93,94]. AQC has also been shown to enhance the ionization yield for MALDI-MS analysis [95]. Although some of the commercial variants of these labeling reagents are not the best solution for high-throughput glycomics at present due to the high cost per sample, they can definitely find their place in the workflows for characterization of therapeutic glycoproteins where number of samples is significantly lower, and time for the analysis is of the essence, especially during the production batch quality control.

Glycans released from the therapeutic glycoproteins which will be separated and analyzed by CGE are most commonly labeled with triply charged APTS, which also allows electrophoretic separation of neutral glycans [96]. The labeling reaction also contains reductants – sodium cyanoborohydride [55,58] or non-toxic 2-picoline borane [97], and acetic or citric acid. APTS glycan labeling with acetic acid usually takes 2 h, which is a trade-off between loss of sialic acids and labeling efficiency [98]. On the other hand, labeling with citric acid for 50 min at 50°C requires 10 times lower consumption of APTS with almost no loss of sialic acids, compared to the incubation at 37°C overnight with acetic acid [99]. An alternative to costly APTS is singly charged 2-amino-1-naphthalenesulfonic acid (2-ANSA), which results in less efficient glycan separation in CGE. However, 2-ANSA labeled glycans are more efficiently ionized in CE-MS [96].

Native, unlabeled glycans can also be analyzed by various techniques, for example, using MALDI-MS in negative reflectron mode or nano-LC/Q-TOF MS system as was demonstrated in a study of recombinant erythropoietin [52].

3.5. Labeled N- and O-glycans clean-up

Purification of fluorescently labeled glycans to remove protein or excess reagents before the glycans analysis by method of choice is an essential step in the glycan characterization workflow. Although paper chromatography [100,101], precipitation [102] and gel-filtration [56,103] have been used for clean-up of labeled glycans, the most commonly used for biologics are different variations of solid-phase extraction (SPE) [52,80,104,105], for example, anion-exchange, porous graphitized carbon (PGC), reverse phase (RP), and hydrophilic chromatography (HILIC). They are usually supplied as part of the glycan release kits.

Technological trends are to increase the throughput, minimize the sample preparation time, and automate as more steps as possible in the sample clean up. In the last decade, workflows have become significantly faster and less labor intensive – from deglycosylation of proteins in SDS-PAGE gels that took 4 days for processing of 96 samples [106], to deglycosylation in solution overnight and fast 1–2 h manual clean-up on the 96-well GH Polypro (GHP) HILIC-SPE plate [104,107,108], or using Hypersep Diol SPE cartridges [81,109]. Integration of the glycan preparation steps and robotization allowed very fast processing of 96 or 384 samples [44,110] in less than a day. Another reported method for *N*-glycan purification on a robotized platform using a PhyTip column packed with polyamide DPA-6S resin allowed the clean-up of 96 samples in 23 min [111]. Automated workflows reduce the hands-on time as well as costs, and exhibit good robustness and reproducibility. Although this has not yet reached the level of a trained analyst, automation in glycoanalytical technologies will improve over time and can be especially useful for routine QC assays for biopharmaceutical industry. Liquid handling stations from Hamilton or Tecan are suitable choices for this automation.

Alternative purification strategies like magnetic beads have also recently been used – glycans are reversibly captured on the surface of carboxyl-coated microparticles and excess of reagents and protein part of a glycoprotein are washed away from the reaction mixture, this technology is employed in the Fast Glycan Labeling and Analysis Kit (SCIEX) designed for biopharmaceuticals [98]. This magnetic bead workflow has been recently automated and applied for *N*-glycosylation analysis of therapeutic antibodies [55].

3.6. Detection of fluorescently labeled N- and O-glycans and structure determination

Liquid chromatography separation of free labeled glycans and their detection by fluorescence allow sensitive relative quantification of glycans from a sample of therapeutic glycoprotein. Separation mechanism is based on hydrophilic interactions and there is a correlation between retention time and glycan size and charge. By running a fluorescently labeled dextran standard (glucose ladder) and assigning the glucose units (GU) to individual glycan peaks, possible glycan structures can be assign to the peaks with specific GU value using GlycoBase and autoGU databases [112,113]. Commercial softwares such as Unifi and MassLynx (Waters Corporation) software can also be applied for use in discovery, development, and quality control.

Similar principles can be applied for the glycan detection and identification by CGE, but with maltodextrin as a standard ladder, used routinely for therapeutic glycoproteins in lot releasing QC and a tool allowing accurate quantitation [114]. Unidentified oligosaccharides peaks are then assigned GU_{CGE} units (glucose units for CGE) by correlating the retention time and length of the sugar oligomer and annotated by comparison of GU_{CGE} values to specific glycan structures in a database. The major disadvantage of CGE method was the lack of standards and very scarce database which could be used for glycan peak annotation. Fortunately, this has been changing and recently a free database with APTS labeled glycans has been developed and is being expanded for use by glycobiologists and the biopharmaceutical

industry [115]. Additionally, while chromatographic methods can easily be coupled with MS for identification of glycan peaks, coupling of CGE and MS is very challenging due to several reasons: gel and buffers used in CGE for glycan separation are not compatible with MS analysis, modification of CGE system in a way that would allow online MS analysis of eluting glycan peaks significantly lowers CGE glycan separation resolution, fluorescent label has to be compatible with both CGE and MS [116,117]. However, a lot of promising work has been done recently to facilitate glycan characterization by connecting CGE separation with MS detection and may be useful for future biologic characterization methods [99,118,119].

Free glycans can also be analyzed by LC-MS using PGC column to separate glycans which are then detected by LC-MS. This PGC-LC-ESI-MS system can be used for glycan isomers separation and detection of *N*- and *O*-glycans both from individual glycoproteins and from complex mixtures [12,52]. Sialylated glycan analysis by MS is usually less quantitative [120] due to the sensitivity of sialic acids to temperature, pH, and ionization conditions, although this may not be a major challenge for many commercial glycoproteins such as mAbs as they do not usually contain a high degree of sialic acids. On the other hand, it could be problematic in characterization of specific highly sialylated therapeutic glycoproteins. Additionally, sialylated glycans easily form salt adducts resulting in multiple signals in the MS spectrum, while carboxyl group due to its negative charge decreases the sialylated glycans signal in MALDI-TOF-MS positive ionization mode. A typical approach is to introduce derivatization step to make sialylated glycans more stable, analysis more sensitive and quantification more robust. Although permethylation [121,122] is most commonly mentioned, the number of transferred methyl groups can vary making the interpretation and analysis more complex. Other carboxylic acid-specific derivatization strategies like methyl esterification [123] and methyl amidation [124] require clean initial glycan sample and harsh derivatization conditions, but also allow differentiation of glycans with α2,6 or α2,3 linked sialic acids. Another similar approach that enables differentiation of α2,6 or α2,3 linked sialic acids is ethyl esterification which uses 1-hydroxybenzotriazole monohydrate and 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide hydrochloride in ethanol to almost completely modify only sialylated glycans [125]. After esterification, glycans are typically cleaned-up using HILIC-SPE on cotton tips, sepharose, or hydrophilic GHP filter [125,126] to remove the excess of reagents before the analysis. Since only α2,6 linked sialic acid effects the anti-inflammatory activity of IgG antibody [127], differentiation of α2,6 from α2,3 sialic acid isomer is of utmost importance in the production of therapeutic antibodies. This derivatization workflow has been automated using liquid-handling robot system that can process 384 samples for the subsequent MALDI-TOF-MS analysis of free *N*-glycans in only 7 h [126].

4. Glycopeptide analysis of therapeutic glycoproteins

Therapeutic glycoprotein analysis on the level of glycopeptides enables site-specific glycosylation analysis, which is a more detailed level of characterization than analysis of released glycans, see Figure 3. It also provides useful structural information on the therapeutic glycoproteins heterogeneity and allows structural characterization of *N*- and *O*-glycopeptides. Therefore, the technique is commonly used in the biopharmaceutical industry during the characterization of the glycoprotein of interest or during drug discovery and recombinant proteins erythropoietin, follicle-stimulating hormone have been characterized using this approach [18,128]. However, it is not usually employed in QC assays as this approach is often more challenging since it requires high-resolution sensitive MS techniques and due to the high costs compared to UPLC/CE techniques used in released *N*- and *O*-glycan characterization. The workflow involves the digestion of the therapeutic glycoprotein with proteases such as trypsin, Lys-C, or Glu-C to give glycopeptide fragments, glycopeptide enrichment, and mass spectrometry techniques such as LC-MS, MALDI, ion mobility, and the increasingly popular MS/MS, see Sections 4.1–4.3.

4.1. Digestion of therapeutic glycoproteins to glycopeptides

The usual approach in glycopeptide analysis is a specific enzymatic digestion of a glycoprotein with a protease, most commonly trypsin, and the reaction is performed overnight at 37°C. Some alternative faster protocols have been described include trypsinization in a microreactor [129] or in a microwave oven [130–132] which may be useful tools for future glycopeptide characterization of therapeutics. In certain circumstances, other proteases are employed either independently or as a cocktail with trypsin such as in a study of recombinant erythropoietin [133]. In this case, trypsin and Glu-C were used in combination. In another example, chymotrypsin was the protease of choice for the recombinant glycoprotein follicle stimulating hormone, which was found to exhibit superior digestion to trypsin [134].

One challenge associated with the use of trypsin with glycoproteins compared to non-glycosylated proteins is that it can result in missed cleavages that are near glycosylation sites due to the steric hindrances from the glycan and produce glycopeptides that are too large for the subsequent MS analysis. Therefore more and more alternative approaches to trypsin digestion for glycopeptide analysis have emerged to overcome this obstacle. For example, the nonspecific proteases (e.g. pronase which contains a mixture of exo- and endo-proteinases) are very useful in glycopeptide analysis because they result in short glycopeptides suitable for MS/MS approaches [135–138]. At present, they are limited to pure proteins preparations only due to the increased heterogeneity of the produced glycopeptides derived from the same site but with different peptide lengths as well as the glycan heterogeneity. This characteristic therefore only poses a minor challenge for therapeutic glycoprotein analysis, with their high degree of purity.

The other approach to evaluate glycan site-specific glycosylation information is to treat the glycoprotein with PNGase F, which converts the Asn residues on the protein to Asp and a shift of one mass unit for each *N*-glycosylation site. Thus, when a deglycosylated protein is further digested with a protease such as trypsin, the peptides that are bound to the glycan moiety will be 1 Da heavier than the expected theoretical mass and the structures can then be characterized using MS [136].

4.2. Glycopeptide enrichment

The relative poor ionization of glycopeptides (and glycoproteins) compared to their non-glycosylated counterparts for MS techniques poses a challenge for the characterization of therapeutic glycoproteins. Additionally, non-glycosylated peptides from the reaction mixture, as well as excess of reagents can further suppress the ionization of glycopeptides, therefore lowering the sensitivity and in turn the relative quantification of glycopeptides for therapeutic glycoprotein characterization. Enrichment and/or purification of glycopeptides after digestion is commonly an integral part of the glycopeptide analysis workflow to improve sensitivity and enhance detection respectively for MS [139]. There are number of different enrichment approaches for glycopeptide analysis applicable for therapeutic glycoprotein characterization described in the literature. Notably, reverse phase C18-SPE which is based on the glycopeptide retention on the stationary phase due to the peptide hydrophobicity [120], or HILIC-SPE [125,140] or HILIC beads [129] which is based on the glycopeptide or glycan partitioning into the water layer on the stationary phase surface are gaining traction. Also, in recent years, more and more workflows are based on glycopeptide binding to functionalized beads [141] or magnetic particles [141,142] to integrate and simplify the steps of glycopeptide preparation for the subsequent analysis. Additionally PGC, lectin, immunoaffinity, hydrazide, boronic acid, or click chemistry enrichment approaches have also been described [18,128,135,138,139] for glycopeptide enrichment. However, at present, HILIC enrichment is the most promising technique for therapeutic glycoproteins as it displays superior separation for glycopeptides and forms the basis for various commercial kits. Commercially available glycopeptides enrichment kits suitable for biopharmaceutical industry include ProteoExtract® Glycopeptide Enrichment Kit (EMD Millipore) and LudgerTag V-TAG glycopeptide labeling and enrichment kit (Ludger Ltd).

4.3. Glycopeptide analysis

Glycopeptide characterization of therapeutic glycoproteins is performed using MS techniques, with identification and structural characterization and quantification afforded using LC-MS, CE-MS, MALDI, ion mobility spectroscopy (IMS), and increasingly tandem mass spectrometry (MS/MS). For quantification, either label-free quantification methods or quantification methods with stable isotope labeling or stable isotope-coded internal standards can be used. These MS techniques allows characterization of *N*- and *O*-glycopeptides and are especially useful in the case for *O*-glycopeptides, as the corresponding released *O*-glycan analysis technologies are currently limited as described in Section 3.3. Representative examples of glycopeptide analysis of therapeutic glycoproteins employing LC-MS [143–145], CE-MS [133], and MALDI [146] are provided and can be suitable technologies for glycopeptide analysis of therapeutic glycoproteins. However, in recent years, there is a trend toward the alternative use of IMS or MS/MS techniques for glycopeptide analysis of therapeutic glycoproteins [128,139].

Simply detecting the m/z values of the glycopeptide fragments of glycoforms is typically not sufficient for high confidence analysis of therapeutic glycoproteins. Instead fragmentation data using MS/MS on the glycoforms should be provided with techniques such as collision-induced dissociation (CID), electron transfer dissociation (ETD), or infrared multiphoton dissociation or combinations thereof, see Figure 3 [128,147,148]. All of these technologies work on the principle that dissociation of the parent molecule/ion into smaller fragments can be used to characterize the precursor molecule/ion and each offer unique strengths and weakness for glycan structural elucidation. No consensus in the field exists to the preferential method for therapeutic glycoproteins. While CID provide information related to the composition of glycan moiety attached to a peptide backbone, ETD allows fragmentation of only peptide backbones while keeping posttranslation modifications intact, thus a combination of the techniques is seen as advantageous for glycopeptide characterization, as demonstrated by Mechref [149]. Higher-energy collisional dissociation (HCD), a CID technique specific to the orbitrap mass spectrometer, is another technique which has shown extremely high resolving power to characterize recombinant proteins, that is, human IgG1 reference material from NIST was characterized using a range of HCD collision energies [150].

The IMS approach for glycopeptide characterization has gained traction due to its ability to resolve different isobaric glycopeptides with identical m/z values but different compositional structures, linkages, or branching [151] such as those containing different NANA linkages [152] and the technique is often used in conjunction with MS/MS techniques. The principle of IMS relies on the separation of biomolecules based on their collisional cross-sectional size through a buffer gas [153]; as such glycan branching, linkages, and structures can be differentiated using the technique. High field asymmetric wave ion mobility spectrometry, also known as differential ion mobility was employed to separate isomeric *O*-linked glycopeptides from the glycoprotein mucin 5AC with identical sequences but differing glycosylation sites [154]. Additionally, traveling wave IMS-MS was used to characterize IgG1 mAb glycosylation heterogeneity profile [155]. One possible shortfall for its application for characterization of therapeutic glycoproteins is the limited availability of the softwares available for IMS data interpretation [156] and improvements are needed in the future.

Bioinformatic tools for the characterization of therapeutic glycoproteins lags far behind the more established omic technologies such as genomics and proteomics. Softwares and bioinformatic tools for glycopeptide characterization of therapeutic glycoproteins are often specialized toward the particular mass spectrometry technique and are reviewed in the literature [157,158]. Broadly speaking there are four classifications of tools available for therapeutic glycoproteins: (1) those that facilitate data input using (high resolution) MS data; (2) those that facilitate data input using MS/MS data; (3) those that allow a combination of MS and MS/MS data input; (4) those that allow IMS data input. These can be further divided into freely available software, of which there are many [156,159–162] and commercial softwares such as SimGlycan, ProSightPC Software, SIEVE Software for Differential Analysis (Thermo Scientific), MassHunter Software (Agilent), Byonic Software (Protein Metrics), or BiopharmaLynx (Waters Corporation).

5. Intact glycoprotein analysis of therapeutic glycoproteins

Intact glycoprotein analysis can be important for detection and separation of heterogeneous glycoforms of an intact therapeutic glycoprotein. Although lectin arrays and HPLC/UPLC methods can be useful tools for intact glycoprotein analysis [18], the vast majority of intact glycoprotein analysis is conducted using mass spectrometry techniques, see Figure 3. Similarly to glycopeptide analysis, enrichment techniques such as CE, HILIC, RP, ion exchange, PGC, lectin, antibody are often employed for intact glycoprotein analysis of therapeutics [163–167]. MS and MS/MS analysis can then accurately assign different glycoforms of therapeutic glycoproteins [18]. As is the case for many IgG mAbs on the market, only Fc glycosylation is present (two sites, see Figure 1) and intact glycoprotein analysis identifies and characterizes ion pairs such as G0F/G0F ($FucGlcNAc_2Man_3GlcNAc_2$ is designated G0F), G0F/G1F ($FucGlcNAc_2Man_3GlcNAc_2Gal$ is designated G1F), G1F/G1F, and G1F/G2F ($FucGlcNAc_2Man_3GlcNAc_2Gal_2$ is designated G2F) for your mAb, see Figure 3 for a representative chromatogram. Analysis of intact monoclonal IgG antibodies have been reported for MS techniques including MALDI [168], electrospray ionization (ESI) source [169], capillary zone electrophoresis-electrospray-mass spectrometry (CZE-ESI-MS) [170], and a combination of mass and top down analyses [164]. MS/MS techniques have the power to give a more comprehensive coverage and ionization techniques such as ETD [171] and CID [164] have been employed in the characterization of intact therapeutic glycoproteins. It should be noted that many more examples of glycopeptide analysis of therapeutic glycoproteins using MS and MS/MS techniques are found in the literature compared to intact glycoprotein however.

Native or near native mass spectrometry using the 'top-down' MS technique is an important tool to characterize intact therapeutic glycoproteins at their pseudo physiological environment and offers many attractive features to the biopharmaceutical industry. The technique allows measurement of glycoproteins in their folded conformation, but also allows dynamic and protein stability studies, as well as assessment of their structural properties [172]. The principle relies on the direct infusion of the biopharmaceutical after buffer exchange into the mass spectrometer and ionization is afforded with soft ionization techniques such as ESI such as Nano-ESI with (quadrupole)-time-of-flight (q)TOF analyzers [172–174]. In the future, the application of native mass spectrometry for characterization of therapeutic glycoproteins will increase with advances in MS because the technology has the capability to identify folding, protein, and glycan structural information as well as relative quantitation in a single experiment. With similar promise for the future applications for therapeutic glycoproteins, IMS, which is finding widespread use in glycopeptide analysis, is quite limited to date with respect to its application in intact glycoprotein analysis, although it has been used for structural elucidation of glycoforms for glycoprotein IgG2 [175].

For data analysis of intact glycoproteins using MS or MS/MS techniques, users can employ many of the software packages described for glycopeptide analysis including the commercial products SimGlycan, ProSightPC Software and SIEVE Software for Differential Analysis (Thermo Scientific), Byonic software (Protein Metrics), MassHunter (Agilent), and MassLynx, BiopharmaLynx (Waters). For native mass spectrometry, ProSightPC Software (Thermo Scientific), Byonics software (Protein Metrics), and MassLynx (Waters) can be employed.

6. Conclusion

Glycoanalytical technologies are essential for characterization of therapeutic glycoproteins for drug discovery, development, and for QC lot and batch variations to ensure safety and efficacy of the biotherapeutic. As discussed in this review, glycosylation analysis can be undertaken implementing various analytical techniques such as LC, CE, and MS techniques or combinations thereof. Recent advances in these technologies have been described with a focus on their (potential) application in the biopharmaceutical industry accordingly and are discussed in the context of their classifications as released glycan analysis, glycopeptide analysis, and intact glycoprotein analysis. Relevant strengths and limitations of each technology toward characterization of therapeutic glycoproteins are described and specific case studies are provided, where possible. In this regard, there is no accepted 'gold standard' standardized approach for therapeutic glycoprotein characterization and instead the industry must use customized methods for characterization of their therapeutic glycoprotein/s. This remains a challenging feat and much needed efforts are required toward the formation of suitable guidelines to overcome some of these hurdles. Nevertheless, the significant potential that novel glycoanalytical technologies hold toward improving the detection of glycans in biotherapeutics, for example, from potentially immunogenic sources will lead to safer and more effective drugs in the future.

7. Expert commentary

Eight out of 10 best selling drugs in Europe are glycoproteins [176] and there is a crucial need for advancement in understanding and characterization of glycosylated biotherapeutics. The manufacture of glycoprotein biologics such as mAbs, cytokines, or therapeutic replacement enzymes is more challenging than for traditional small molecule drugs. Minor alterations in the production process can dramatically alter the efficacy or immunogenicity of the drug product and posttranslation modifications such as glycosylation can often play a dominant role. Consequently, strict requirements are necessary for validation, lot release, and batch release of glycosylated biotherapeutics to satisfy regulatory bodies. To this end, many biopharmaceutical companies already have well-established glycoanalytical platforms for their biotherapeutics characterization. However, the balance between maintaining established glycoanalytical tools and the development of novel glycoanalytical technologies will need to be adjusted to accommodate new advances in the field toward ultimately producing safer and more efficacious biologics. This will require participation from both industry, regulatory bodies and governmental agencies whereby incentives are provided to update current platforms to use state of the art glycoanalytical technologies, both for their established products and

those that are emerging. For the novel glycoanalytical methods, the focus should be to develop fast, cheap, automated, robust, and user friendly methods while maintaining the requirements set out by the regulatory requirements.

A recent trend is the increasing emphasis on understanding the nature of the glycans of the therapeutic glycoprotein. Given that various cell lines, expression hosts and protocols can result in different glycosylation patterns [25], measuring and understanding glycosylation using analytical techniques is crucial. With improvements such as increased sensitivity for glycan detection and upgrades in the state of the art instrumentation, analytical techniques now allow lower levels of detection of glycans than in the past. This proves particularly important for the biopharmaceutical field toward the identification and reduction of particular epitopes that may be immunogenic, such as α-Gal or Neu5Gc; also known as NGNA. Additionally, MS advances including native mass spectrometry and IMS are particularly exciting and will provide additional structural information of the therapeutic glycoproteins. As summarized throughout this review, accumulated reports clearly indicate strong evidence that emerging glycoanalytical approaches will successfully contribute toward developing safer and more efficacious biotherapeutics.

8. Five-year view

The use of glycoanalytical approaches with respect to biopharmaceuticals will continue to expand during discovery, in the clinics and in manufacturing phases of biopharmaceuticals with continuous development of more sensitive instrumentation, methodology, and novel commercial applications. One existing problem remains how to develop and expand new technologies for the characterization of released *O*-glycans of therapeutic glycoproteins. *O*-glycopeptide analysis on the other hand is far more developed but is not suitable for many QC assays. Unlike *N*-glycans, no global enzyme has been found to date to successfully digest all *O*-glycan structures in glycoproteins and as such chemical methods are currently employed. Persisting problems with undesirable peeling products, the selectivity of these methods and a need for chemical expertise hampers their analyses. A novel *O*-glycan global enzyme would solve the problem. However, in its absence, standardized, user friendly approaches and novel technologies are needed to overcome some of these shortcomings in the future.

Ongoing efforts for automation and standardization of typical glycoanalytical workflows for glycoprotein characterization enable high throughput analyses [81,110], and thus drive the field of glycomics toward more clinical applications and standardized approaches useful for the biopharmaceutical industry, particularly for QC assays where robustness and reproducibility will likely improve with increased automation. Glycosylation Critical Quality Attribute criteria will tighten for biopharmaceuticals as the technologies improve and a deeper understanding of the biological effects is known. Moreover, the glycomics toolbox will enlarge with the growing interest in the field with advances in glycoengineering of biologics for improved immune functions [25], improvements in intact glycoprotein analyses, and aggregation studies for glycan heterogeneity and macroscale considerations [18]. There is also a recent focus on personalized medicines, of which glycoanalytical technologies for their characterization will be developed. To conclude, the recent and upcoming developments in the glycoanalytical technologies and their employment in the analysis of therapeutic glycoproteins are most exciting for the future and will aid the pharmaceutical industry to exploit the full potential of their biopharmaceuticals. However, there is much room for improvement with regard to standardization of glycoanalytical techniques and the development of a glycoanalytical technique for glycomics synonymous with CRISP-Cas for genomics that address all the different aspects important for characterization of biotherapeutics.

Key issues

- Glycosylation of a therapeutic glycoprotein is a Critical Quality Attribute. At present, it is difficult to obtain a full complement of structural information from one single technique. Therefore a combination of released glycan, glycopeptide and intact glycoprotein profiling is required to structurally characterize the protein sequence, glycan moieties and glycosylation sites of a therapeutic glycoprotein.
- Glycoanalytical techniques are often cumbersome, laborious and manual and at present need expertise of a trained analyst. Automation and robotization will increase throughput and enhance robustness of glycoanalytical methods.
- Therapeutic glycoproteins are expressed in various expression systems such as CHO or murine myeloma cell lines and the role of glycosylation can be complex. The association between protein glycosylation and specific immune function can be poorly understood and can result in adverse immune reactions. Additionally, many antibody drug conjugates and fusion proteins are being developed by biopharmaceutical industry. There is a need for special consideration and techniques to minimize any undesired immune responses in humans and to extent current analytical technologies to expand these new classes of biotherapeutics.
- Improvements in sensitivity and resolution of glycoanalytical technologies is needed for detection of low abundant glycoforms. Development and expansion of analytical technologies for released *O*-glycan characterization of therapeutic glycoproteins for QC assays is especially needed also.
- Bioinformatic tools, software packages and data analysis in the field of glycomics lags far behind proteomics or genomics and development of commercial packages which allow data input (and manipulation and visualization) from released glycan, glycopeptide and glycoprotein experiments using various source inputs for orthogonal characterization of therapeutics is strongly needed.

Acknowledgements

R.O.F. and P.M.R. acknowledges EU FP7 program HighGlycan, grant no. 278535, for funding this work. I.T.A and G.L. acknowledges EU FP7 program MIMOmics, grant no. 305280; HTP-GlycoMet, grant no. 324400; PainOmics, grant no. 602736; H2020 grants GlySign, grant no. 722095;

GlyCoCan, grant no. 676412; SYSCID, grant no. 733100 and IMforFuture, grant no. 721815 as well as funding for the Croatian National Centre of Research Excellence in Personalized Healthcare. G.G. acknowledge funding from Science foundation Ireland Starting Investigator Research grant (SFI SIRG) under grant number 13/SIRG/2164.

Funding

R. O'Flaherty and P.M. Rudd would like to acknowledge EU FP7 program HighGlycan, grant no. 278535, for funding this work. I. Trbojevic-Akmacic and G.Lauc would like to acknowledge EU FP7 program MIMOmics, grant no. 305280; HTP-GlycoMet, grant no. 324400; PainOmics, grant no. 602736; EU H2020 grants GlySign, grant no. 722095; GlyCoCan, grant no. 676412; SYSCID, grant no. 733100 and IMforFuture, grant no. 721815 as well as funding for the Croatian National Centre of Research Excellence in Personalized Healthcare. G.Greville would like to acknowledge funding from Science Foundation Ireland Starting Investigator Research grant (SFI SIRG) under grant number 13/SIRG/2164.

Declaration of interest

G. Lauc is the founder and owner of Genos Ltd, a private research organization that specializes in high-throughput glycomic analysis and has several patents in this field. I. Trbojević-Akmačić is the employee of Genos Ltd. 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.

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References

Papers of special note have been highlighted as either of interest (•) or of considerable interest (••) to readers.

1. Apweiler R, Hermjakob H, Sharon N. On the frequency of protein glycosylation, as deduced from analysis of the SWISS-PROT database. Biochim Biophys Acta. 1999;1473(1):4–8.
2. Huang T, Chen X, Gu H, *et al*. Fractionation of Fab glycosylated immunoglobulin G with concanavalin A chromatography unveils new structural properties of the molecule. Oncotarget. 2016;7(21):31166–31176.
• **The first identification of four N-glycan sites for the Fab region on IgG is described.**
3. Varki A, Cummings RD, Aebi M, *et al*. Symbol nomenclature for graphical representations of glycans. Glycobiology. 2015;25(12):1323–1324. •• **SNFG nomenclature is the glycan nomenclature used in this review for visualization of released glycans and glycopeptides.**
4. Dell A, Galadari A, Sastre F, *et al*. Similarities and differences in the glycosylation mechanisms in prokaryotes and eukaryotes. Int J Microbiol. 2010;2010:148178.
5. Moremen KW, Tiemeyer M, Nairn AV. Vertebrate protein glycosylation: diversity, synthesis and function. Nat Rev Mol Cell Biol. 2012;13(7):448–462.
6. Cutler A, Brombacher F. Cytokine therapy. Ann N Y Acad Sci. 2005;1056:16–29.
7. Song K, Yoon IS, Kim NA, *et al*. Glycoengineering of interferon-beta 1a improves its biophysical and pharmacokinetic properties. PLoS One. 2014;9(5):e96967.
8. Kagawa Y, Takasaki S, Utsumi J, *et al*. Comparative study of the asparagine-linked sugar chains of natural human interferon-beta 1 and recombinant human interferon-beta 1 produced by three different mammalian cells. J Biol Chem. 1988;263(33):17508–17515.
9. Pastores GM. Agalsidase alfa (Replagal) in the treatment of Anderson-Fabry disease. Biologics. 2007;1(3):291–300.
10. Kizhner T, Azulay Y, Hainrichson M, *et al*. Characterization of a chemically modified plant cell culture expressed human alpha-galactosidase-A enzyme for treatment of Fabry disease. Mol Genet Metab. 2015;114(2):259–267.
11. Freeze H, Haltiwanger RS. Other classes of ER/Golgi-derived glycans. In Varki A, Cummings R, Esko JD, et al. Ed. Essentials of glycobiology. *2nd*. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press; 2009.
12. Jensen PH, Karlsson NG, Kolarich D, *et al*. Structural analysis of N- and O-glycans released from glycoproteins. Nat Protoc. 2012;7(7):1299–1310.
•• **A protocol for the characterization of N- and O-glycans from glycoproteins is described.**
13. Negrier C, Young G, Abdul Karim F, *et al*. Recombinant long-acting glycoPEGylated factor IX (nonacog beta pegol) in haemophilia B: assessment of target joints in multinational phase 3 clinical trials. Haemophilia. 2016;22(4):507–513.
14. DeFrees S, Wang ZG, Xing R, *et al*. GlycoPEGylation of recombinant therapeutic proteins produced in *Escherichia coli*. Glycobiology. 2006;16(9):833–843.
15. Tarp MA, Clausen H. Mucin-type O-glycosylation and its potential use in drug and vaccine development. Biochim Biophys Acta. 2008;1780(3):546–563.
16. Cheong TC, Compagno M, Chiarle R. Editing of mouse and human immunoglobulin genes by CRISPR-Cas9 system. Nat Commun. 2016;7:10934.
17. Ronda C, Pedersen LE, Hansen HG, *et al*. Accelerating genome editing in CHO cells using CRISPR Cas9 and CRISPy, a web-based target finding tool. Biotechnol Bioeng. 2014;111(8):1604–1616.
18. Zhang L, Luo S, Zhang B. Glycan analysis of therapeutic glycoproteins. MAbs. 2016;8(2):205–215.
19. Geyer H, Geyer R. Strategies for analysis of glycoprotein glycosylation. Biochim Biophys Acta. 2006;1764(12):1853–1869.
20. Wakankar A, Chen Y, Gokarn Y, *et al*. Analytical methods for physicochemical characterization of antibody drug conjugates. MAbs. 2011;3(2):161–172.
21. Tian Y, Zhang H. Characterization of disease-associated N-linked glycoproteins. Proteomics. 2013;13(3–4):504–511.
22. Ecker DM, Jones SD, Levine HL. The therapeutic monoclonal antibody market. MAbs. 2015;7(1):9–14.
23. U.S. Food and Drug Administration [Internet] Silver Spring (MD 20993): FDA; [cited 2017 September 30]. Available from: https://www.fda.gov/drugs/developmentapprovalprocess/howdrugsaredevelopedandapproved/approvalapplications/therapeuticbiologicapplications/biosimilars/ucm411418.htm
24. Tang Y, Tang F, Yang Y, *et al*. Real-time analysis on drug-antibody ratio of antibody-drug conjugates for synthesis, process optimization, and quality control. Sci Rep. 2017;7(1):7763.
•• **This publication describes a real time drug antibody ratio analysis of antibody drug conjugates for therapeutic efficacy and pharmacokinetics studies. This type of study will be increasingly important for the expanding ADCs market and may be incorporated into glycoanalytical characterization in the future.**
25. Mimura Y, Katoh T, Saldova R, *et al*. Glycosylation engineering of therapeutic IgG antibodies: challenges for the safety, functionality and efficacy. Protein Cell; 2017; DOI:10.1007/s13238-017-0433-3.
26. Bandaranayake AD, Almo SC. Recent advances in mammalian protein production. FEBS Lett. 2014;588(2):253–260.
27. Bosques CJ, Collins BE, Meador JW 3rd, *et al*. Chinese hamster ovary cells can produce galactose-alpha-1,3-galactose antigens on proteins. Nat Biotechnol. 2010;28(11):1153–1156.
28. Jenkins N, Parekh RB, James DC. Getting the glycosylation right: implications for the biotechnology industry. Nat Biotechnol. 1996;14(8):975–981.
29. Kunert R, Reinhart D. Advances in recombinant antibody manufacturing. Appl Microbiol Biotechnol. 2016;100(8):3451–3461.

30. Hossler P, Khattak SF, Li ZJ. Optimal and consistent protein glycosylation in mammalian cell culture. Glycobiology. 2009;19(9):936–949.
31. Keservani RK, Sharma AK, Kumar Kesharwani R. Novel approaches for drug delivery. Advances in Medical Technologies and Clinical Practise (AMTCP); 2016;Hershey,PA (U.S.A); IGI Global .
32. Mellstedt H. Clinical considerations for biosimilar antibodies. EJC Suppl. 2013;11(3):1–11.
33. Mullard A. 2016 FDA drug approvals. Nat Rev Drug Discov. 2017;16 (2):73–76.
34. Easton R. Analysis of glycosylation in biosimilars. Biopharm Int. 2016;29(8):45–49.
35. Grampp G, Ramanan S. The diversity of biosimilar design and development: implications for policies and stakeholders. Biodrugs. 2015;29(6):365–372.
36. Chung CH, Mirakhur B, Chan E, *et al*. Cetuximab-induced anaphylaxis and IgE specific for galactose-alpha-1,3-galactose. N Engl J Med. 2008;358(11):1109–1117.
37. Higel F, Seidl A, Sörgel F, *et al*. N-glycosylation heterogeneity and the influence on structure, function and pharmacokinetics of monoclonal antibodies and Fc fusion proteins. Eur J Pharmaceutics Biopharmaceutics. 2016;100(Supplement C):94–100.
38. World Health Organisation Similiar biotherapeutic [Internet]. Geneva (Switzerland): World Health Organisation; [cited 2017 September 30]. Available from: http://www.who.int/biologicals/biotherapeutics/similar_biotherapeutic_products/en/
39. European Medicines Agency ICH Q6B specifications: test procedures and acceptance criteria for biotechnological/biological products [Internet]. London (U.K.): European Medicines Agency; [cited 2017 September 30]. Available from: http://www.ema.europa.eu/ema/index.jsp?curl=pages/regulation/general/general_content_000883.jsp&mid=
40. Costa S, Almeida A, Castro A, *et al*. Fusion tags for protein solubility, purification and immunogenicity in *Escherichia coli*: the novel Fh8 system. Front Microbiol. 2014;5:63.
41. Totten SM, Kullolli M, Pitteri SJ. Multi-lectin affinity chromatography for separation, identification, and quantitation of intact protein glycoforms in complex biological mixtures. Methods Mol Biol. 2017;1550:99–113.
42. Stöckmann H, O'Flaherty R, Adamczyk B, *et al*. Automated, high-throughput serum glycoprofiling platform. Integr Biol. 2015;7 (9):1026–1032.
43. Chen M, Lu Y, Ma Q, *et al*. Boronate affinity monolith for highly selective enrichment of glycopeptides and glycoproteins. Analyst. 2009;134(10):2158–2164.
44. Stöckmann H, Duke RM, Millán Martín S, *et al*. Ultrahigh throughput, ultrafiltration-based n-glycomics platform for ultraperformance liquid chromatography (ULTRA3). Anal Chem. 2015;87 (16):8316–8322.
45. Liu HF, Ma J, Winter C, *et al*. Recovery and purification process development for monoclonal antibody production. mAbs. 2010;2 (5):480–499.
46. Hinderer W, Arnold S. Process for the purification of recombinant human erythropoietin (epo), epo thus purified and pharmaceutical compositions comprising same. (Google Patents, 2010)
47. Arnau J, Lauritzen C, Petersen GE, *et al*. Current strategies for the use of affinity tags and tag removal for the purification of recombinant proteins. Protein Expr Purif. 2006;48(1):1–13.
48. Saraswat M, Musante L, Ravida A, *et al*. Preparative purification of recombinant proteins: current status and future trends. Biomed Res Int. 2013;2013:312709.
49. Choe W, Durgannavar TA, Chung SJ. Fc-binding ligands of immunoglobulin G: an overview of high affinity proteins and peptides. Materials (Basel). 2016;9(12).
50. Zhang S, Daniels W, Salm J, *et al*. Nature of foulants and fouling mechanism in the Protein A Mabselect resin cycled in a monoclonal antibody purification process. Biotechnol Bioeng. 2016;113(1):141–149.
51. Yoshimura T, Yamada G, Narumi M, *et al*. Detection of N-glycans on small amounts of glycoproteins in tissue samples and sodium dodecyl sulfate-polyacrylamide gels. Anal Biochem. 2012;423 (2):253–260.
52. Oh MJ, Hua S, Kim BJ, *et al*. Analytical platform for glycomic characterization of recombinant erythropoietin biotherapeutics and biosimilars by MS. Bioanalysis. 2013;5(5):545–559.
53. Jmeian Y, Hammad L, Fast MY. Efficient online release of N-glycans from glycoproteins facilitating liquid chromatography–tandem mass spectrometry glycomic profiling. Anal Chem. 2012;84:8790–8796.
54. Krenkova J, Szekrenyes A, Keresztessy Z, *et al*. Oriented immobilization of peptide-N-glycosidase F on a monolithic support for glycosylation analysis. J Chromatogr. 2013;1322:54–61.
55. Szigeti M, Lew C, Roby K, *et al*. Fully automated sample preparation for ultrafast N-glycosylation analysis of antibody therapeutics. J Lab Autom. 2016;21:281–286.
56. Melmer M, Stangler T, Schiefermeier M, *et al*. HILIC analysis of fluorescence-labeled N-glycans from recombinant biopharmaceuticals. Anal Bioanal Chem. 2010;398(2):905–914.
57. Zhou H, Briscoe AC, Froehlich JW, *et al*. PNGase F catalyzes de-N-glycosylation in a domestic microwave. Anal Biochem. 2012;427 (1):33–35.
58. Szabo Z, Guttman A, Karger BL. Rapid release of N-linked glycans from glycoproteins by pressure-cycling technology. Anal Chem. 2010;82(6):2588–2593.
59. Triguero A, Cabrera G, Royle L, *et al*. Chemical and enzymatic N-glycan release comparison for N-glycan profiling of monoclonal antibodies expressed in plants. Anal Biochem. 2010;400(2):173–183.
60. Yamamoto K. Microbial endoglycosidases for analyses of oligosaccharide chains. J Biochem. 1994;116:229–235.
61. Bondt A, Rombouts Y, Selman MHJ, *et al*. IgG Fab glycosylation analysis using a new mass spectrometric high-throughput profiling method reveals pregnancy-associated changes. Mol Cell Proteomics. 2014;13(11):3029–3039.
62. Rombouts Y, Willemze A, Van Beers JJBC, *et al*. Extensive glycosylation of ACPA-IgG variable domains modulates binding to citrullinated antigens in rheumatoid arthritis. Ann Rheum Dis. 2016;75 (3):578-585.
63. Mahan AE, Tedesco J, Dionne K, *et al*. A method for high-throughput, sensitive analysis of IgG Fc and Fab glycosylation by capillary electrophoresis. J Immunol Methods. 2015;417:34–44.
64. Lynaugh H, Li H, Gong B. Rapid Fc glycosylation analysis of Fc fusions with IdeS and liquid chromatography mass spectrometry. MAbs. 2013;5(5):641–645.
65. Kannicht C, Kroning M, Solecka-Witulska BA, *et al*. Comparative N-glycosylation analysis of the fc portions of a chimeric human coagulation factor VIII and immunoglobulin G1. Bioengineering (Basel). 2017;4(2):E44.
66. Moelleken J, Endesfelder M, Gassner C, *et al*. GingisKHAN protease cleavage allows a high-throughput antibody to Fab conversion enabling direct functional assessment during lead identification of human monoclonal and bispecific IgG1 antibodies. MAbs. 2017;9(7):1076-1087.
67. O'Flaherty R, Harbison AM, Hanley PJ, *et al*. Aminoquinoline fluorescent labels obstruct efficient removal of N-glycan core alpha(1–6) fucose by bovine kidney alpha-L-fucosidase (BKF). J Proteome Res. 2017;16(11):4237–4243.
68. Marino K, Bones J, Kattla JJ, *et al*. A systematic approach to protein glycosylation analysis: a path through the maze. Nat Chem Biol. 2010;6(10):713–723.
• **Exoglycosidase digestion of N-glycans can be employed as an orthogonal technique for released glycan, glycopeptide or intact glycoprotein characterization of therapeutic glycoproteins.**
69. Thomson RI, Gardner RA, Strohfeldt K, *et al*. Analysis of three epoetin alpha products by LC and LC-MS indicates differences in glycosylation critical quality attributes, including sialic acid content. Anal Chem. 2017;89(12):6455–6462.
70. Nix DB, Kumagai T, Katoh T, *et al*. Improved in-gel reductive beta-elimination for comprehensive O-linked and sulfo-glycomics by mass spectrometry. J Vis Exp. 2014;93:e51840.
71. Houel S, Hilliard M, Yu YQ, *et al*. N- and O-glycosylation analysis of etanercept using liquid chromatography and quadrupole time-of-

flight mass spectrometry equipped with electron-transfer dissociation functionality. Anal Chem. 2014;86(1):576–584.
72. Kim U, Oh MJ, Seo Y, *et al*. Sensitive and comprehensive analysis of O-glycosylation in biotherapeutics: a case study of novel erythropoiesis stimulating protein. Bioanalysis. 2017;9(18):1373–1383.
73. Patel TP, Parekh RB. Release of oligosaccharides from glycoproteins by hydrazinolysis. Methods Enzymol. 1994;230:57–66.
74. Patel T, Bruce J, Merry A, *et al*. Use of hydrazine to release in intact and unreduced form both N- and O-linked oligosaccharides from glycoproteins. Biochemistry. 1993;32(2):679–693.
75. Turyan I, Hronowski X, Sosic Z, *et al*. Comparison of two approaches for quantitative O-linked glycan analysis used in characterization of recombinant proteins. Anal Biochem. 2014;446:28–36.
76. Morelle W, Michalski JC. Analysis of protein glycosylation by mass spectrometry. Nat Protoc. 2007;2(7):1585–1602.
77. Magnelli PE, Bielik AM, Guthrie EP. Identification and characterization of protein glycosylation using specific endo- and exoglycosidases. J Vis Exp. 2011;58:3749.
78. Deguchi K, Keira T, Yamada K, *et al*. Two-dimensional hydrophilic interaction chromatography coupling anion-exchange and hydrophilic interaction columns for separation of 2-pyridylamino derivatives of neutral and sialylated N-glycans. J Chromatogr. 2008;1189 (1–2):169–174.
79. Anumula KR, Dhume ST. High resolution and high sensitivity methods for oligosaccharide mapping and characterization by normal phase high performance liquid chromatography following derivatization with highly fluorescent anthranilic acid. Glycobiology. 1998;8(7):685–694.
80. Ruhaak LR, Huhn C, Waterreus WJ, *et al*. Hydrophilic interaction chromatography-based high-throughput sample preparation method for N-glycan analysis from total human plasma glycoproteins. Anal Chem. 2008;80(15):6119–6126.
81. Stockmann H, O'Flaherty R, Adamczyk B, *et al*. Automated, high-throughput serum glycoprofiling platform. Integr Biol (Camb). 2015;7(9):1026–1032.
• **An example of an automated glycoanalytical technology to increase throughput, reproducibility and robustness for characterization of therapeutic glycoproteins.**
82. Klapoetke S, Zhang J, Becht S, *et al*. The evaluation of a novel approach for the profiling and identification of N-linked glycan with a procainamide tag by HPLC with fluorescent and mass spectrometric detection. J Pharm Biomed Anal. 2010;53(3):315–324.
83. Sato K, Sato K, Okubo A, *et al*. Separation of 2-aminobenzoic acid-derivatized glycosaminoglycans and asparagine-linked glycans by capillary electrophoresis. Anal Sci. 2005;21:21–24, Copyright (C) 2012 U.S. National Library of Medicine.
84. Kamoda S, Ishikawa R, Kakehi K. Capillary electrophoresis with laser-induced fluorescence detection for detailed studies on N-linked oligosaccharide profile of therapeutic recombinant monoclonal antibodies. J Chromatogr. 2006;1133(1–2):332–339.
85. Prien JM, Prater BD, Qin Q, *et al*. Mass spectrometric-based stable isotopic 2-aminobenzoic acid glycan mapping for rapid glycan screening of biotherapeutics. Anal Chem. 2010;82 (4):1498–1508.
86. Gil G-C, Iliff B, Cerny R, *et al*. High throughput quantification of N-Glycans using one-pot sialic acid modification and matrix assisted laser desorption ionization time-of-flight mass spectrometry. Anal Chem. 2010;82(15):6613–6620.
87. Balog C, Stavenhagen K, Fung WLJ, *et al*. N-glycosylation of colorectal cancer tissues: a liquid chromatography and mass spectrometry-based investigation. Mol Cell Proteomics. 2012;11(9):571–585.
88. Xie H, Chakraborty A, Ahn J, *et al*. Rapid comparison of a candidate biosimilar to an innovator monoclonal antibody with advanced liquid chromatography and mass spectrometry technologies. MAbs. 2010;2(4):379–394.
89. Harvey DJ. Electrospray mass spectrometry and fragmentation of N-linked carbohydrates derivatized at the reducing terminus. J Am Soc Mass Spectrom. 2000;11(10):900–915.
90. Higel F, Demelbauer U, Seidl A, *et al*. Reversed-phase liquid-chromatographic mass spectrometric N-glycan analysis of biopharmaceuticals. Anal Bioanal Chem. 2013;405(8):2481–2493.
91. Anumula KR. Advances in fluorescence derivatization methods for high-performance liquid chromatographic analysis of glycoprotein carbohydrates. Anal Biochem. 2006;350(1):1–23.
92. Kozak RP, Tortosa CB, Fernandes DL, *et al*. Comparison of procainamide and 2-aminobenzamide labeling for profiling and identification of glycans by liquid chromatography with fluorescence detection coupled to electrospray ionization-mass spectrometry. Anal Biochem. 2015;486:38–40.
93. Lauber MA, Yu Y-Q, Brousmiche DW, *et al*. Rapid preparation of released N-glycans for HILIC analysis using a labeling reagent that facilitates sensitive fluorescence and ESI-MS detection. Anal Chem. 2015;87(10):5401–5409.
94. Bosch L, Alegría A, Farré R. Application of the 6-aminoquinolyl-N-hydroxysccinimidyl carbamate (AQC) reagent to the RP-HPLC determination of amino acids in infant foods. J Chromatogr B: anal Technologies Biomed Life Sci. 2006;831(1–2):176–183.
95. Ullmer R, Plematl A, Rizzi A. Derivatization by 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate for enhancing the ionization yield of small peptides and glycopeptides in matrix-assisted laser desorption/ionization and electrospray ionization mass spectrometry. Rapid Commun Mass Spe. 2006;20:1469–1479.
96. Bunz SC, Cutillo F, Neusüß C. Analysis of native and APTS-labeled N-glycans by capillary electrophoresis/time-of-flight mass spectrometry. Anal Bioanal Chem. 2013;405(25):8277–8284.
97. Ruhaak LR, Steenvoorden E, Koeleman C, *et al*. 2-Picoline-borane: a non-toxic reducing agent for oligosaccharide labeling by reductive amination. Proteomics. 2010;10:2330–2336.
98. Váradi C, Lew C, Guttman A. Rapid magnetic bead based sample preparation for automated and high throughput N-glycan analysis of therapeutic antibodies. Anal Chem. 2014;86(12):5682–5687.
99. Szabo Z, Guttman A, Rejtar T, *et al*. Improved sample preparation method for glycan analysis of glycoproteins by CE-LIF and CE-MS. Electrophoresis. 2010;31(8):1389–1395.
100. Royle L, Radcliffe CM, Dwek R, *et al*. Detailed structural analysis of N-glycans released from glycoproteins in SDS-PAGE gel bands using HPLC combined with exoglycosidase array digestions. Methods Mol Biol. 2006;347:125–143.
101. Kalay H, Ambrosini M, Van Berkel PHC, *et al*. Online nanoliquid chromatography-mass spectrometry and nanofluorescence detection for high-resolution quantitative N-glycan analysis. Anal Biochem. 2012;423(1):153–162.
102. Pabst M, Kolarich D, Pöltl G, *et al*. Comparison of fluorescent labels for oligosaccharides and introduction of a new postlabeling purification method. Anal Biochem. 2009;384(2):263–273.
103. Nakagawa H, Hato M, Takegawa Y, *et al*. Detection of altered N-glycan profiles in whole serum from rheumatoid arthritis patients. J Chromatogr B: anal Technologies Biomed Life Sci. 2007;853(1570–0232):133–137.
104. Trbojević Akmačić I, Ugrina I, Štambuk J, *et al*. High throughput glycomics: optimization of sample preparation. Biochemistry (Moscow). 2015;80(7):934–942.
105. Ruhaak LR, Hennig R, Huhn C, *et al*. Optimized workflow for preparation of APTS-labeled N-glycans allowing high-throughput analysis of human plasma glycomes using 48-channel multiplexed CGE-LIF. J Proteome Res. 2010;9:6655–6664.
106. 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. 2008;376:1–12.
107. Burnina I, Hoyt E, Lynaugh H, *et al*. A cost-effective plate-based sample preparation for antibody N-glycan analysis. J Chromatogr. 2013;1307:201–206.
108. Yu YQ, Gilar M, Kaska J, *et al*. A rapid sample preparation method for mass spectrometric characterization of N-linked glycans. Rapid Commun Mass Spe. 2005;19(16):2331–2336.
109. Adamczyk B, Stockmann H, O'Flaherty R, *et al*. High-throughput analysis of the plasma N-glycome by UHPLC. Methods Mol Biol. 2017;1503:97–108.

110. Stöckmann H, Adamczyk B, Hayes J, *et al*. Automated, high-throughput IgG-antibody glycoprofiling platform. Anal Chem. 2013;85(18):8841–8849.
111. Prater BD, Anumula KR, Hutchins JT. Automated sample preparation facilitated by PhyNexus MEA purification system for oligosaccharide mapping of glycoproteins. Anal Biochem. 2007;369(2):202–209.
112. Campbell MP, Royle L, Radcliffe CM, *et al*. GlycoBase and autoGU: tools for HPLC-based glycan analysis. Bioinformatics. 2008;24(9):1214–1216.
113. Walsh I, O'Flaherty R, Rudd PM. Bioinformatics applications to aid high-throughput glycan profiling. Perspect Sci. 2017;11:31–39.
114. Mittermayr S, Guttman A. Influence of molecular configuration and conformation on the electromigration of oligosaccharides in narrow bore capillaries. Electrophoresis. 2012;33(6):1000–1007.
115. Jarvas G, Szigeti M, Guttman A. GUcal: an integrated application for capillary electrophoresis based glycan analysis. Electrophoresis. 2015;36(24):3094-3096.
116. Bunz SC, Rapp E, Neusüss C. Capillary electrophoresis/mass spectrometry of APTS-labeled glycans for the identification of unknown glycan species in capillary electrophoresis/laser- induced fluorescence systems. Anal Chem. 2013;85(21):10218–10224.
117. Mittermayr S, Bones J. Unraveling the glyco-puzzle: glycan structure identification by capillary electrophoresis. Anal Chem. 2013;85:4228–4238.
118. Maxwell EJ, Ratnayake C, Jayo R, *et al*. A promising capillary electrophoresis-electrospray ionization-mass spectrometry method for carbohydrate analysis. Electrophoresis. 2011;32(16):2161–2166.
119. Jayo RG, Thaysen-Andersen M, Lindenburg PW, *et al*. Simple capillary electrophoresis-mass spectrometry method for complex glycan analysis using a flow-through microvial interface. Anal Chem. 2014;86(13):6479–6486.
120. Huffman JE, Pučić-Baković M, Klarić L, *et al*. Comparative performance of four methods for high-throughput glycosylation analysis of immunoglobulin G in genetic and epidemiological research. Mol Cell Proteomics. 2014;13:1598–1610.
121. Morelle W, Michalski J-C. Analysis of protein glycosylation by mass spectrometry. Nat Protoc. 2007;2(7):1585–1602.
122. Desantos-Garcia JL, Khalil SI, Hussein A, *et al*. Enhanced sensitivity of LC-MS analysis of permethylated N-glycans through online purification. Electrophoresis. 2011;32(24):3516–3525.
123. Miura Y, Shinohara Y, Furukawa JI, *et al*. Rapid and simple solid-phase esterification of sialic acid residues for quantitative glycomics by mass spectrometry. Chem Eur J. 2007;13(17):4797–4804.
124. Liu X, Qiu H, Kuo Lee R, *et al*. Methylamidation for sialoglycomics by MALDI-MS: a facile derivatization strategy for both α2,3- and α2,6-linked sialic acids. Anal Chem. 2010;82:8300–8306.
125. Reiding KR, Blank D, Kuijper DM, *et al*. High-throughput profiling of protein N-glycosylation by MALDI-TOF-MS employing linkage-specific sialic acid esterification. Anal Chem. 2014;86:5784–5793.
126. Bladergroen MR, Reiding KR, Hipgrave Ederveen AL, *et al*. Automation of high-throughput mass spectrometry-based plasma *N*-glycome analysis with linkage-specific sialic acid esterification. J Proteome Res. 2015;14(9):4080–4086.
127. Anthony RM, Nimmerjahn F, Ashline DJ, *et al*. Recapitulation of IVIG anti-inflammatory activity with a recombinant IgG Fc. Science. 2008;320:373–376.
128. Desaire H. Glycopeptide analysis, recent developments and applications. Mol Cell Proteomics. 2013;12(4):893–901.
129. Zhu J, Wang F, Chen R, *et al*. Centrifugation assisted microreactor enables facile integration of trypsin digestion, hydrophilic interaction chromatography enrichment, and on-column deglycosylation for rapid and sensitive N-glycoproteome analysis. Anal Chem. 2012;84(11):5146–5153.
130. Vaezzadeh AR, Deshusses JMP, Waridel P, *et al*. Accelerated digestion for high-throughput proteomics analysis of whole bacterial proteomes. J Microbiol Methods. 2010;80(1):56–62.
131. Komatsu E, Buist M, Roy R, *et al*. Characterization of immunoglobulins through analysis of N-glycopeptides by MALDI-TOF MS. Methods. 2016;104:170-181.
132. Reddy PM, Hsu WY, Hu JF, *et al*. Digestion completeness of microwave-assisted and conventional trypsin-catalyzed reactions. J Am Soc Mass Spectrom. 2010;21(3):421–424.
133. Gimenez E, Ramos-Hernan R, Benavente F, *et al*. Analysis of recombinant human erythropoietin glycopeptides by capillary electrophoresis electrospray-time of flight-mass spectrometry. Anal Chim Acta. 2012;709:81–90.
134. Grass J, Pabst M, Chang M, *et al*. Analysis of recombinant human follicle-stimulating hormone (FSH) by mass spectrometric approaches. Anal Bioanal Chem. 2011;400(8):2427–2438.
    • **A glycopeptide analysis study of FSH therapeutic glycoprotein conducted using chymotrypsin as protease instead of the more commonly employed trypsin.**
135. An HJ, Froehlich JW, Lebrilla CB. Determination of glycosylation sites and site-specific heterogeneity in glycoproteins. Curr Opin Chem Biol. 2009;13(4):421–426.
136. Stavenhagen K, Plomp R, Wuhrer M. Site-specific protein N- and O-glycosylation analysis by a c18-porous graphitized carbon-liquid chromatography-electrospray ionization mass spectrometry approach using pronase treated glycopeptides. Anal Chem. 2015;87(23):11691–11699.
137. Zhu Z, Desaire H. Carbohydrates on proteins: site-specific glycosylation analysis by mass spectrometry. Annu Rev Anal Chem. 2015;8(1):463–483.
138. Kolarich D, Jensen PH, Altmann F, *et al*. Determination of site-specific glycan heterogeneity on glycoproteins. Nat Protoc. 2012;7(7):1285–1298.
139. Zhu R, Zacharias L, Wooding KM, *et al*. Glycoprotein enrichment analytical techniques: advantages and disadvantages. Methods Enzymol. 2017;585:397–429.
140. Selman MHJ, Hemayatkar M, Deelder AM, *et al*. Cotton HILIC SPE microtips for microscale purification and enrichment of glycans and glycopeptides. Anal Chem. 2011;83(7):2492–2499.
141. Zhang Y, Yu M, Zhang C, *et al*. Highly selective and ultra fast solid-phase extraction of N-glycoproteome by oxime click chemistry using aminooxy-functionalized magnetic nanoparticles. Anal Chem. 2014;86:7920–7924.
    • **The employment of functionalized beads for glycopeptide purification as an alternative to traditional plate enrichment techniques is described for characterization of glycoproteins.**
142. Zhang Y, Yu M, Zhang C, *et al*. Highly specific enrichment of N-glycoproteome through a nonreductive amination reaction using Fe_3O_4@SiO_2-aniline nanoparticles. Chem Commun. 2015;51(27):5982–5985.
143. Chevreux G, Tilly N, Bihoreau N. Fast analysis of recombinant monoclonal antibodies using IdeS proteolytic digestion and electrospray mass spectrometry. Anal Biochem. 2011;415(2):212–214.
144. Stadlmann J, Pabst M, Kolarich D, *et al*. Analysis of immunoglobulin glycosylation by LC-ESI-MS of glycopeptides and oligosaccharides. Proteomics. 2008;8(14):2858–2871.
145. Reusch D, Haberger M, Falck D, *et al*. Comparison of methods for the analysis of therapeutic immunoglobulin G Fc-glycosylation profiles—part 2: mass spectrometric methods. MAbs. 2015;7(4):732–742.
146. De Haan N, Reiding KR, Haberger M, *et al*. Linkage-specific sialic acid derivatization for MALDI-TOF-MS profiling of IgG glycopeptides. Anal Chem. 2015;87(16):8284–8291.
147. Hakansson K, Chalmers MJ, Quinn JP, *et al*. Combined electron capture and infrared multiphoton dissociation for multistage MS/MS in a Fourier transform ion cyclotron resonance mass spectrometer. Anal Chem. 2003;75(13):3256–3262.
148. Zeng WF, Liu MQ, Zhang Y, *et al*. pGlyco: a pipeline for the identification of intact N-glycopeptides by using HCD-and CID-MS/MS and MS3. Scientific Reports. 2016;6(25102):1-10.
    •• **A technology is presented combining data from two orthogonal MS/MS techniques for glycopeptide analysis: HCD and CID MS/MS to give a more in depth characterization of glycoproteins.**
149. Mechref Y. Use of CID/ETD mass spectrometry to analyze glycopeptides. Curr Protoc Protein Sci. 2012;0 12:Unit–12.1111.

150. Dong Q, Yan XJ, Liang YX, *et al*. Enhanced glycosylation detection and characterization of a human IgG1 reference material by using 2D-LC MS/MS fractionation and high resolution mass spectral library. Faseb J. 2017;31(1) Supplement 785.5:.
151. Cao L, Qu Y, Zhang ZR, *et al*. Intact glycopeptide characterization using mass spectrometry. Expert Rev Proteomics. 2016;13(5):513–522.
152. Hinneburg H, Hofmann J, Struwe WB, *et al*. Distinguishing N-acetylneuraminic acid linkage isomers on glycopeptides by ion mobility-mass spectrometry. Chem Commun (Camb). 2016;52 (23):4381–4384.
153. Lanucara F, Holman SW, Gray CJ, *et al*. The power of ion mobility-mass spectrometry for structural characterization and the study of conformational dynamics. Nat Chem. 2014;6(4):281–294.
154. Creese AJ, Cooper HJ. Separation and identification of isomeric glycopeptides by high field asymmetric waveform ion mobility spectrometry. Anal Chem. 2012;84(5):2597–2601.
•• **Ion mobility spectroscopy for glycopeptide analysis is presented for characterization of glycoprotein mucin 5AC and can be translated to characterize therapeutic glycoproteins.**
155. Olivova P, Chen W, Chakraborty AB, *et al*. Determination of N-glycosylation sites and site heterogeneity in a monoclonal antibody by electrospray quadrupole ion-mobility time-of-flight mass spectrometry. Rapid Commun Mass Spectrom. 2008;22(1):29–40.
156. Xia D, Ghali F, Gaskell SJ, *et al*. Software for analysing ion mobility mass spectrometry data to improve peptide identification. Proteomics. 2012;12(12):1912–1916.
157. Tsai P-L, Chen S-F. A brief review of bioinformatics tools for glycosylation analysis by mass spectrometry. Mass Spectrom. 2017;6(2):S0064.
158. Walsh I, Zhao S, Campbell M, *et al*. Quantitative profiling of glycans and glycopeptides: an informatics' perspective. Curr Opin Struct Biol. 2016;40:70–80.
159. Cooper CA, Gasteiger E, Packer NH. GlycoMod - a software tool for determining glycosylation compositions from mass spectrometric data. Proteomics. 2001;1(2):340–349.
160. Campbell MP, Nguyen-Khuong T, Hayes CA, *et al*. Validation of the curation pipeline of UniCarb-DB: building a global glycan reference MS/MS repository. Biochim Biophys Acta. 2014;1844(1 Pt A):108–116.
161. Woodin CL, Hua D, Maxon M, *et al*. GlycoPep grader: a web-based utility for assigning the composition of N-linked glycopeptides. Anal Chem. 2012;84(11):4821–4829.
162. MacLean B, Tomazela DM, Shulman N, *et al*. Skyline: an open source document editor for creating and analyzing targeted proteomics experiments. Bioinformatics. 2010;26(7):966–968.
163. Ahn YH, Kim JY, Yoo JS. Quantitative mass spectrometric analysis of glycoproteins combined with enrichment methods. Mass Spectrom Rev. 2015;34(2):148–165.
164. Bondarenko PV, Second TP, Zabrouskov V, *et al*. Mass measurement and top-down HPLC/MS analysis of intact monoclonal antibodies on a hybrid linear quadrupole ion trap-Orbitrap mass spectrometer. J Am Soc Mass Spectrom. 2009;20(8):1415–1424.
165. Tetaz T, Detzner S, Friedlein A, *et al*. Hydrophilic interaction chromatography of intact, soluble proteins. J Chromatogr A. 2011;1218 (35):5892–5896.
166. Haselberg R, De Jong GJ, Somsen GW. CE-MS for the analysis of intact proteins 2010–2012. Electrophoresis. 2013;34(1):99–112.
167. Pedrali A, Tengattini S, Marrubini G, *et al*. Characterization of intact neo-glycoproteins by hydrophilic interaction liquid chromatography. Molecules. 2014;19(7):9070–9088.
168. Alexander AJ, Hughes DE. Monitoring of IgG antibody thermal stability by micellar electrokinetic capillary chromatography and matrix-assisted laser desorption/ionization mass spectrometry. Anal Chem. 1995;67(20):3626–3632.
169. Bennett KL, Smith SV, Lambrecht RM, *et al*. Rapid characterization of chemically-modified proteins by electrospray mass spectrometry. Bioconjug Chem. 1996;7(1):16–22.
170. Balaguer E, Neususs C. Glycoprotein characterization combining intact protein and glycan analysis by capillary electrophoresis-electrospray ionization-mass spectrometry. Anal Chem. 2006;78 (15):5384–5393.
171. Fornelli L, Damoc E, Thomas PM, *et al*. Analysis of intact monoclonal antibody IgG1 by electron transfer dissociation Orbitrap FTMS. Mol Cell Proteomics. 2012;11(12):1758–1767.
172. Thompson NJ, Rosati S, Heck AJ. Performing native mass spectrometry analysis on therapeutic antibodies. Methods. 2014;65(1):11–17.
•• **Representative native mass spectrometry approach for characterization of intact glycoprotein for measurement of glycoproteins in their folded conformation, dynamic and protein stabillity and assessment of structural properties.**
173. Rose RJ, Damoc E, Denisov E, *et al*. High-sensitivity Orbitrap mass analysis of intact macromolecular assemblies. Nat Methods. 2012;9 (11):1084–1086.
174. Yang Y, Liu F, Franc V, *et al*. Hybrid mass spectrometry approaches in glycoprotein analysis and their usage in scoring biosimilarity. Nat Commun. 2016;7(13397).
175. Bagal D, Valliere-Douglass JF, Balland A, *et al*. Resolving disulfide structural isoforms of IgG2 monoclonal antibodies by ion mobility mass spectrometry. Anal Chem. 2010;82(16):6751–6755.
176. Roadmap for glycoscience in Europe [Internet]; [cited 2017 September 30]. Available from (http://www.ibcarb.com/glycoscienceroadmap/index.html