

AUTOMATED DETECTION AND IDENTIFICATION OF *N*- AND *O*-GLYCOPEPTIDES

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ABSTRACT

Because it can provide detailed information about aglycons, glycosylation sites, and the composition and structure of glycans, mass spectrometry is highly suited for the analysis of glycopeptides and released *N*- and *O*-glycans. Here we present the bioinformatics platform ProteinScape, which can process entire LC-MS/MS runs, localise spectra that contain glycan-related information, and perform searches against glycan structure databases. An intuitive user interface facilitates interactive validation of results. If glycans are not released and glycopeptides are analysed, the heterogeneity of glycosylation at the various protein glycosylation sites can be assessed.

The integration of protein- and glycan-related functionality in a single software platform is particularly useful not only in glycoproteomics research, but also in biopharmaceutical development and QC. We provide several examples illustrating the efficiency of glycopeptide analysis using mass spectrometry. However, a comprehensive analysis requires information on the glycoprotein's mass profile. Therefore, the interpretation of mass spectra from intact glycoproteins is also discussed.

INTRODUCTION

Glycosylation is one of the most common and important post-translational protein modifications. Glycoproteins have diverse functions and are involved in numerous biological processes. Their glycan moieties are either directly involved in regulatory processes or influence physicochemical properties of the glycoprotein. In eukaryotes, the vast majority of secreted and plasma membrane proteins are known to be glycosylated and glycosylation has been shown to be important for various protein functions [1]. The significance of glycoproteins is further demonstrated by the fact that more than 90% of proteins produced for therapeutic purposes – such as antibodies or hormones – are glycoproteins. In such biologics, glycosylation has been demonstrated to control parameters such as serum half-life or receptor interaction kinetics (e. g., [2]).

As a consequence, protein analysis projects – from large-scale proteomics down to focused protein characterisation studies – often require a detailed study of glycosylation. This is particularly true for the characterisation and quality control of recombinant biopharmaceutical proteins. However, due to a lack of dedicated and easy-to-use software, glycosylation is often neglected in the proteomics community. To address this issue, we have extended the scope of our bioinformatics software to the analysis of glycosylation.

ProteinScape is Bruker Daltonics' central bioinformatics platform for storage and processing of MS data. It supports various gel- and LC-based workflows. The underlying database organises all relevant data for all types of proteomics projects – including LC-data, gel data, mass spectra, process parameters and search results. ProteinScape acts as a central control unit and data evaluation tool for mass spectrometry-based identification, characterisation, and quantitation. The data hierarchy of the software is an accurate reflection of laboratory workflows – with projects, samples, separations, fractions; MS data and search results forming the main hierarchy levels. Information is accessible in tables and viewers and through extensive query functionalities. Dedicated viewers enable fast and interactive evaluation and validation of data and results (Figure 1). Real-time updating of the linked displays speeds up browsing and information management. As ProteinScape is able to handle data from different mass spectrometers, it is the ideal tool to study protein glycosylation.

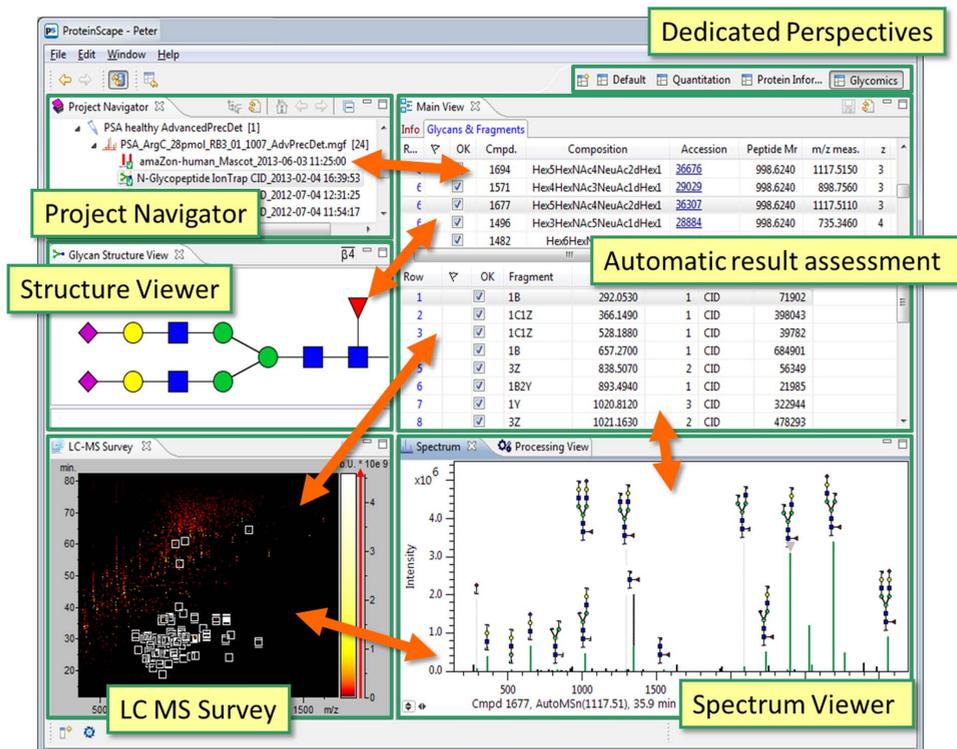


Figure 1. The ProteinScape user interface consists of closely linked navigators, tables, and data viewers. In addition to preset application-specific “Perspectives” (protein ID, protein quantitation, protein information, and glycomics), the arrangement of views can be freely adapted to the requirements of a specific workflow and saved as a user-defined “Perspective”.

THREE STRATEGIES FOR GLYCOPROTEIN ANALYSIS

Three strategies can be applied for the comprehensive characterisation of a glycosylated protein. Figure 2 summarises these strategies.

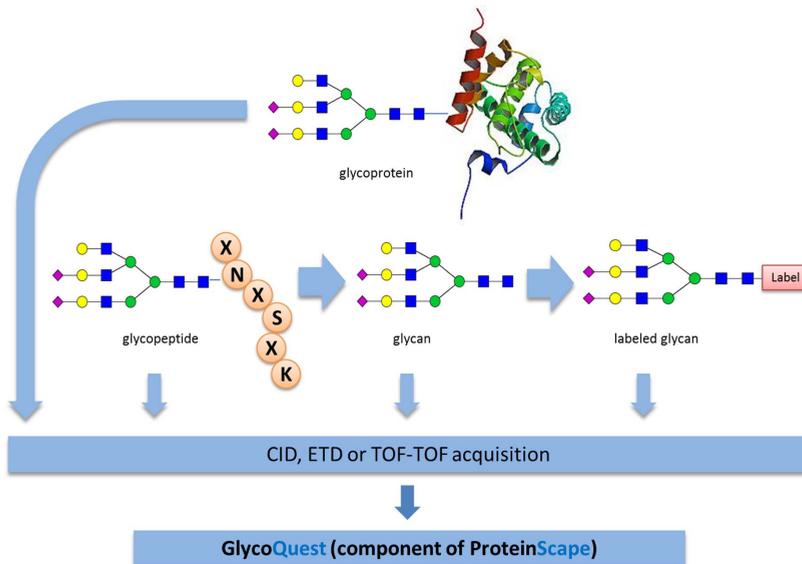


Figure 2. Glycoproteins can be analysed either as intact molecules, or after proteolytic digestion. Glycans can be analysed either as part of the respective glycopeptide, or as enzymatically- or chemically-released glycans that can be labelled using a fluorophore for optical detection in LC separations.

Strategy 1 is the separation of glycans from the protein or peptides. This common workflow generates a peptide fraction – that can be used for protein identification and the evaluation of other modifications – and a glycan fraction – that can be used for the identification of all glycans originally attached to the protein.

Compared to a proteolytic digest of a glycoprotein, the glycan fraction has a lower complexity and can be further separated and analysed by glycan-specific methods. The glycans can either be submitted to mass spectrometry without further modification, or a label (e.g., a fluorophore for visual detection) can be introduced to the reducing end of the glycans, which are then analysed by LC and/or mass spectrometry.

Strategy 2 is the proteolytic digestion of intact glycoproteins. If glycans are released, the information about the initial attachment of the respective glycans to the glycosylation sites of the individual proteins is lost. Proteolytic digestion of glycoproteins yields glycopeptides that can provide important information about the actual state of the various glycosylation sites.

Strategy 3 is the analysis of intact glycoproteins. Here, the heterogeneity of glycosylation is retained with the highest fidelity.

Strategy 1: Analysis of released glycans

ProteinScape's integrated processing pipeline contains several powerful algorithms: The GlycoQuest search engine is particularly useful for automated glycan analysis. Glycans released from the protein by a peptide-*N*-glycosidase (*N*-glycans) or by reductive beta-elimination (*O*-glycans) are automatically detected, identified, and reported.

As with protein database searches, glycan search parameters are defined and stored in a search method (Figure 3). Data are then searched against a user-defined glycan database or the meta-database GlycomeDB (<http://www.glycome-db.org>), which was developed at the German Cancer Research Center in Heidelberg and currently contains around 39,000 glycan structures. GlycomeDB is regularly updated with structures from various primary databases and automatically synchronised with GlycoQuest. Searches can be restricted to a primary database. A glycan structure editor enables manual editing of glycan structures that can be saved to a user-defined database for GlycoQuest searches. Figures 4 and 5 show two exemplary search results.

The screenshot shows the GlycoQuest method editor interface. It is a web-based form with various input fields and dropdown menus. The main sections are:

- Glycan type:** N-Glycan
- Database:** CarbBank
- Taxonomy:** Homo sapiens
- Composition:** H0-9, N0-6, S0-3, F0-3
- Modifications:** Sulfate, N-Sulfate, O-methyl, Phosphate
- Derivatization:** UND
- Reducing end:** AA
- Ions:** H+, Na+, K+, Li+
- H+ up to:** 1
- Neutrals:** Water, Ammonia
- Charge +:** 1 to 1
- Charge -:** 1 to 1
- MS tolerance (m/z):** 50 ppm
- MS/MS tolerance (m/z):** 0.3 Da
- Fragmentation type CID:** CID a b c x y z
- ETD:** ETD
- Threshold for glycan list compilation:**
 - Score >: 10
 - Fragmentation coverage [%] >: 10
 - Intensity coverage [%] >: 30

Figure 3. The GlycoQuest method editor. Parameters include one of GlycomeDB's primary databases (e.g. CarbBank), the glycan type (e.g. *N*-glycan), allowed compositions, and mass tolerances. Searches can be started for an individual spectrum, for an LC-MS/MS run, or for batches of spectra or LC-MS/MS runs.

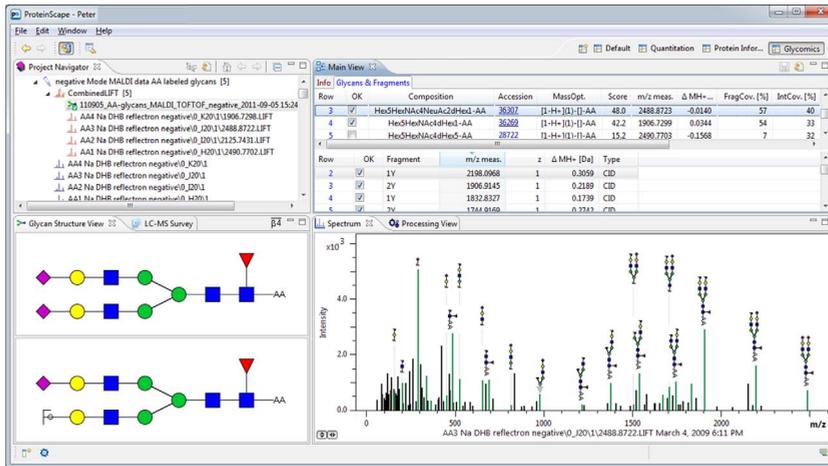


Figure 4. GlycoQuest result: a MALDI spectrum of a 2-aminobenzoic acid (AA)-labelled *N*-glycan, acquired on an ultrafleXtreme MALDI-TOF instrument.

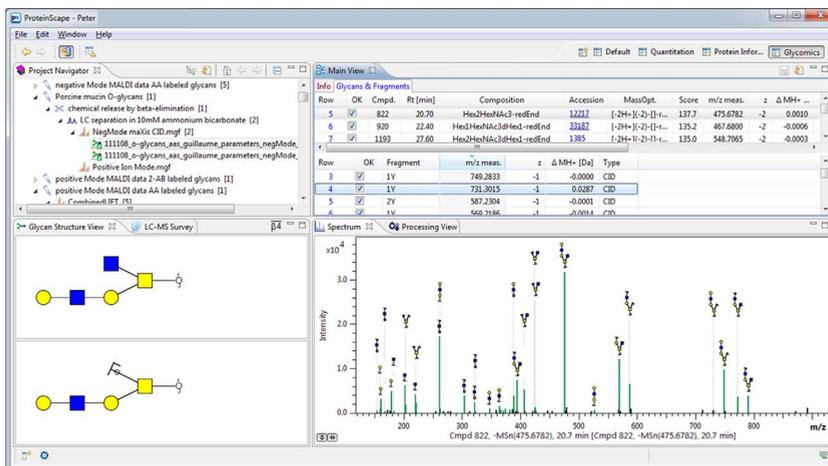


Figure 5. GlycoQuest result: an ESI spectrum of a reduced *O*-glycan, acquired on a maXis impact ESI-TOF instrument.

It should be noted here that a complete confirmation of a glycan's structure – which is highly dependent on the availability of all diagnostic fragment signals in the MS/MS spectrum – is a difficult task. GlycoQuest is an algorithm that compares spectra with theoretical fragment patterns. Some isomeric structures cannot be distinguished using this method. The situation becomes even more complicated if different glycans are not separated, and isobaric structures yield a mixture of fragments in the MS/MS spectrum. A satisfactory analysis of isomeric structures can be achieved by a variety of dedicated methods, such as permethylation of the sample, followed by the acquisition of MSⁿ spectra in an ion trap [3].

An alternative approach is the separation of reduced glycans by porous graphitised carbon chromatography. This technique is able to separate glycans that differ in structure but have the same composition and, therefore, the same mass. This allows differentiation of glycans with subtle linkage differences. For example, neuraminic acid [α 2,3 or α 2,6], galactose [β 1,4 or α 1,3] and the two G1 isomers that are frequently found on IgG *N*-glycans can be separated. In many cases the MS/MS spectra from such structures, although yielding identical theoretical fragments, have fundamental differences [4–7].

Strategy 2: Glycopeptide analysis

Glycopeptide analysis is a challenging task [8]. Due to high glycan heterogeneity and ion suppression effects, abundance of glycopeptide signals from proteolytic digests is usually low, and specific enrichment and separation techniques might be required. In addition, interpretation of MS/MS spectra is difficult as classical database search approaches cannot be used if the peptide's and the glycan's molecular weights are unknown. The correct determination of the peptide mass is a crucial feature for automated glycopeptide identification.

The glycopeptide classifier

The task of finding glycopeptide spectra in an LC-MS/MS dataset of a digested glycoprotein sample is demanding. ProteinScape searches for characteristic patterns in the MS/MS spectra and submits only relevant spectra to the database. Because both Mascot (Matrix Science) and GlycoQuest perform precursor-based searches, the exact masses of the glycan and peptide moieties of each glycopeptide must be known beforehand. The glycopeptide classifier algorithm of ProteinScape uses characteristic mass patterns (MALDI) or fragment ion series (ESI CID) to determine the glycan and peptide moiety masses. In this way, the glycopeptide classifier is mandatory for a successful identification of glycopeptides. Examples for *N*-glycopeptides are given in Figures 6 and 7. However, the glycopeptide classifier is not limited to *N*-glycopeptides. For example, core-fucosylated *N*-glycopeptides and also several kinds of *O*-glycopeptides in MALDI and ESI spectra can also be handled.

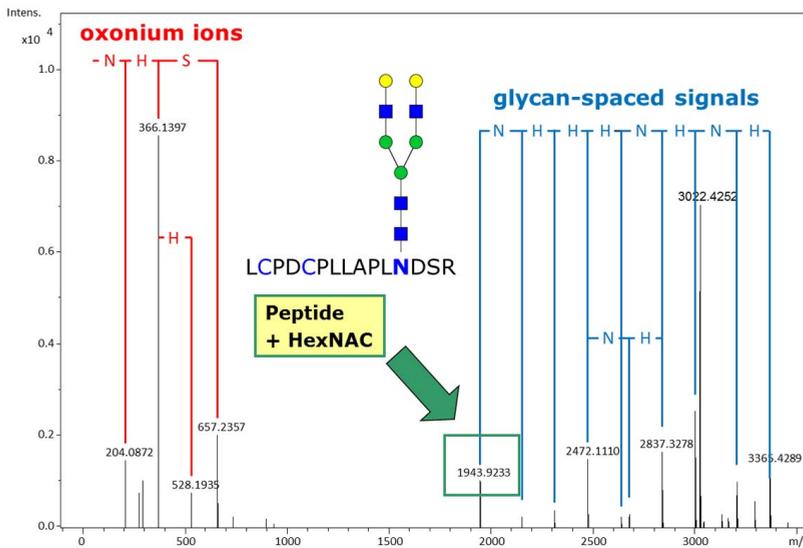


Figure 6. Deconvoluted ESI-CID spectra of *N*-glycopeptides usually contain diagnostic mass signals in the low molecular weight range, plus a characteristic tree of fragment ions that can be followed down to the mass of the peptide plus one *N*-acetylglucosamine.

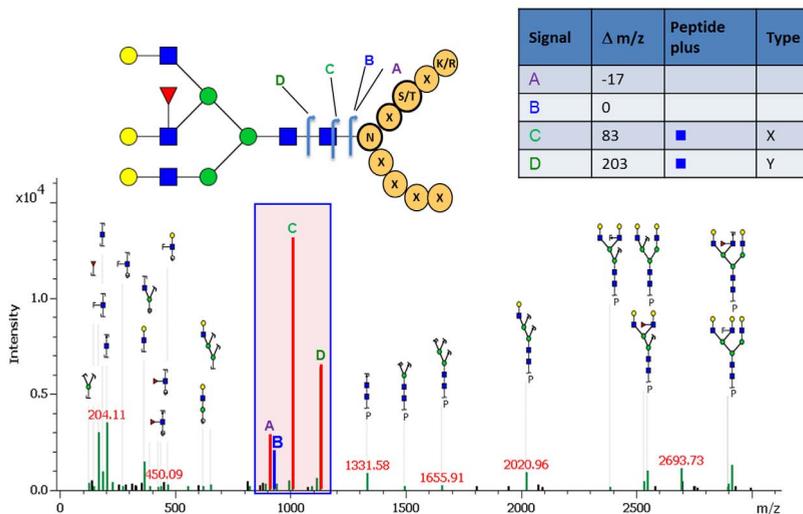


Figure 7. MALDI-TOF/TOF spectra of *N*-glycosylated peptides contain a specific fragment pattern. A cleavage between the glycan and peptide part produces the MH^+ of the peptide. The *N*-acetylglucosamine attached to the asparagine undergoes Y and $^{0,2}X$ cross-ring fragmentation providing strong fragment ions that are 83 and 203 Da heavier. A loss of ammonia from the asparagine completes the pattern ([8]).

Example: EPO

An example of the characterisation of a glycoprotein by the identification of glycopeptides is our study [9] on Erythropoietin (EPO). EPO is a glycoprotein with hormone activity that controls the production of red blood cells in bone marrow. Recombinant human EPO is produced on a large scale in cell culture as a therapeutic agent for treating anaemia related to different diseases. It is also abused as a blood doping agent in endurance sports. Human EPO is an approximately 34 kDa glycoprotein with one *O*- and three *N*-glycosylation sites (Figure 8). In pharmaceutical drug production, glycosylation heterogeneity of EPO is an important quality factor that influences functionality as well as bioavailability of the therapeutic protein.

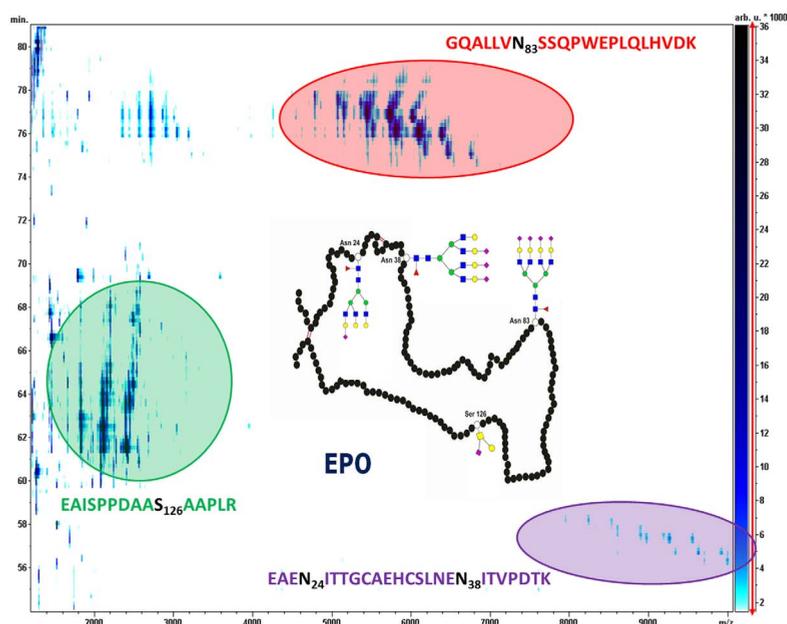


Figure 8. Survey view of an LC-MALDI-MS experiment on an EPO-BRP tryptic digest. Singly charged peptides (m/z) are displayed against retention time (min). Three glycopeptide fractions are visible: Two *N*-linked glycopeptide fractions (red and purple) and one *O*-linked glycopeptide fraction (green). The four glycosylation sites of EPO are one *O*-glycosylated serine (S126), and three *N*-glycosylated asparagines (N24, N38 and N83). A particular difficulty arises from the fact that in a tryptic digest, N24 and N38 are found in the same peptide.

EPO BRP (LGC Standards) and recombinant human EPO expressed in HEK 293 cells (Sigma-Aldrich) were reduced, alkylated and subjected to trypsin digestion. The resulting peptides and glycopeptides were separated by nano-HPLC (nano-Advance, Bruker) and further analysed by MALDI-TOF/TOF-MS (ultrafleXtreme, Bruker). MS/MS spectra were imported into ProteinScape 3.1 and automatically screened for an *O*-glycopeptide-specific

fragmentation pattern. The typical pattern for core 1 structures ($-18/+203/+162$) was used to detect the relevant MS/MS spectra and to determine the peptide moiety mass of each glycopeptide. Subsequently, protein database searches were performed using Mascot (Matrix Science). GlycoQuest was used for glycan identification in GlycomeDB and in a custom-made EPO database that also contained acetylated structures not present in GlycomeDB (Figure 9). It could be shown that the glycosylation profile of EPO expressed in HEK 293 cells is much more heterogeneous than that of the BRP standard, and that the degree of acetylation is greatly reduced (Figure 10).

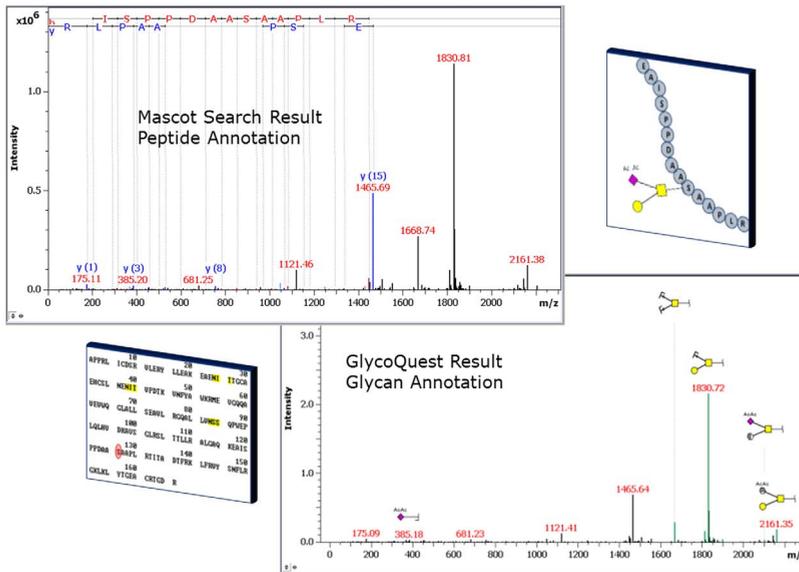


Figure 9. Mascot and GlycoQuest result for the spectrum of one *O*-glycopeptide. MALDI TOF/TOF spectra of *O*-linked glycopeptides contain both peptide and glycan fragments.

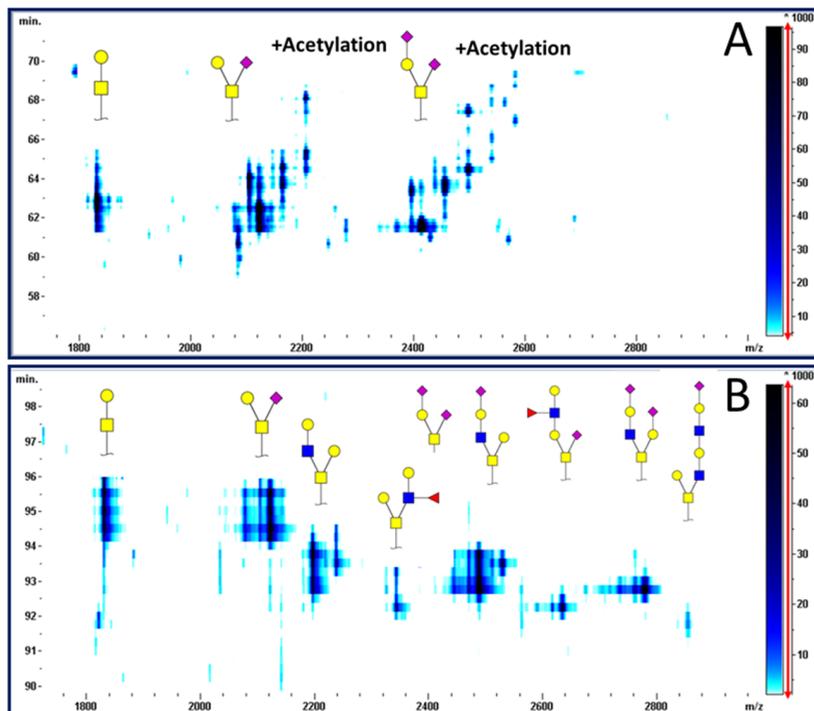


Figure 10. The Survey Viewer zoomed into the *O*-linked glycopeptide range of EPO BRP-Standard (A) and EPO HEK 293 cells (B) enables an at-a-glance comparison. Main differences were found in terms of glycan forms and acetylation grade.

Strategy 3: Glycoprotein analysis

Example 1: Cetuximab, a recombinant antibody

Because they play a central role in the immune response of vertebrates, antibodies represent one of the most important classes of glycoproteins. There is a growing interest in recombinant antibodies as biotherapeutic agents. Such so-called biologics are manufactured in biological systems, and usually employ recombinant technology. These systems can be very sensitive to minor changes in the manufacturing process, which may significantly alter the final biologic product. They can introduce modifications that may adversely affect the safety and efficacy of the drug. Therefore, strict quality control of each biopharmaceutical batch and comparison with reference standards is essential to ensure reproducibility between batches and to achieve regulatory approval.

Cetuximab is a chimeric mouse-human IgG1 that targets the epidermal growth factor receptor (EGFR). It is approved for use in the EU and US as a treatment for colorectal cancer and squamous cell carcinoma of the head and neck. The amino acid sequences for both the light and heavy chains of cetuximab (Figure 11) are reported in the IMGT database (<http://www.imgt.org>) and the drug bank (www.drugbank.ca). A high prevalence of hypersensitivity reactions to cetuximab were reported in some areas of the US. Different glycoforms were shown to be responsible for these hypersensitivity reactions and anaphylaxis. This example demonstrates the importance of a precise analysis of glycosylation in biopharmaceutical proteins.

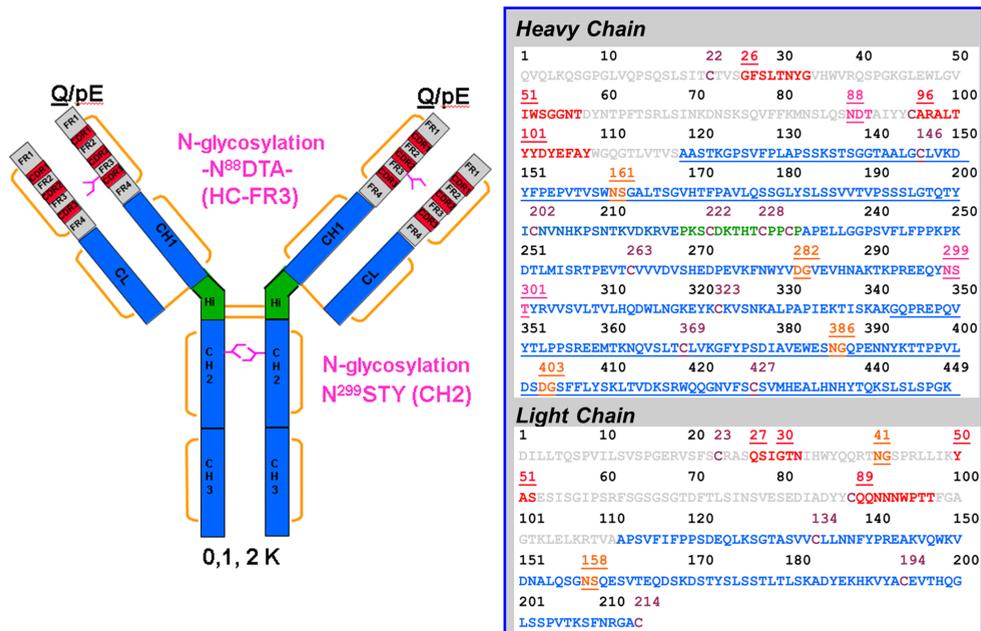


Figure 11. Schematic structure and sequence of cetuximab. Colours indicate protein QC “hot spots”. Of particular importance are the two heavy-chain glycosylation sites, which can be separated from each other by proteolytic cleavage in the hinge region.

In the study of Ayoub *et al.* [10], detailed sequence information of the antibody’s subunits was obtained using MALDI *N*- and *C*-terminal top-down sequencing (TDS) analysis. LC-MS/MS peptide mapping experiments on tryptic and GluC digests enabled post-translational modifications and sequence variants to be further localised.

For intact-mass analysis, the two heavy-chain *N*-glycosylation sites were separated by enzymatic cleavage (Figure 12). The LC-ESI mass spectra of the cetuximab subunits (middle-up approach) yielded glycosylation site-specific accurate masses of the various antibody glycoforms. Using GlycoQuest, glycopeptide and glycan identifications and

profiles were automatically generated. Finally, the results from middle-up data were combined with the results from the bottom-up glycopeptide identification to generate a complete picture of the antibody's glycosylation (Figures 13 – 15).

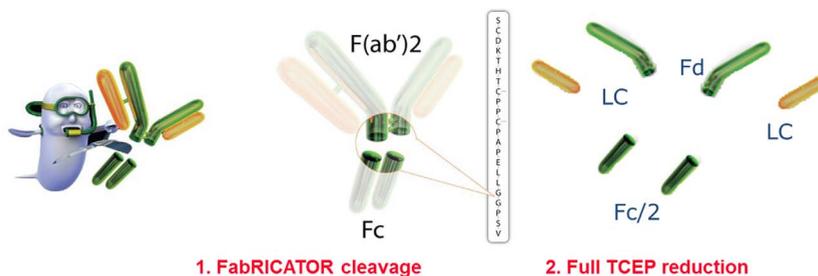


Figure 12. Middle-up approach: a dedicated proteolytic enzyme (FabRICATOR, Genovis) cleaves the heavy chain at the conserved Gly–Gly motif in the hinge region, thereby separating the two glycosylation sites [11], (Figure adapted from www.genovis.com).

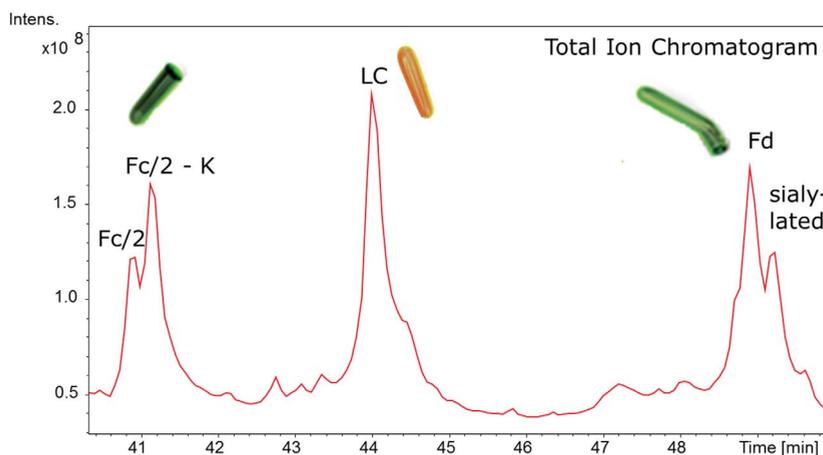


Figure 13. The light chain and both fragments of the heavy chain are separated by RP HPLC. The chromatogram shows a heterogeneous sialylation of the *N*-terminal Fd fragment (already indicating different glycosylation profiles for both sites) and lysine-clipping of the C-terminal Fc fragment, (Figure adapted from [10]).

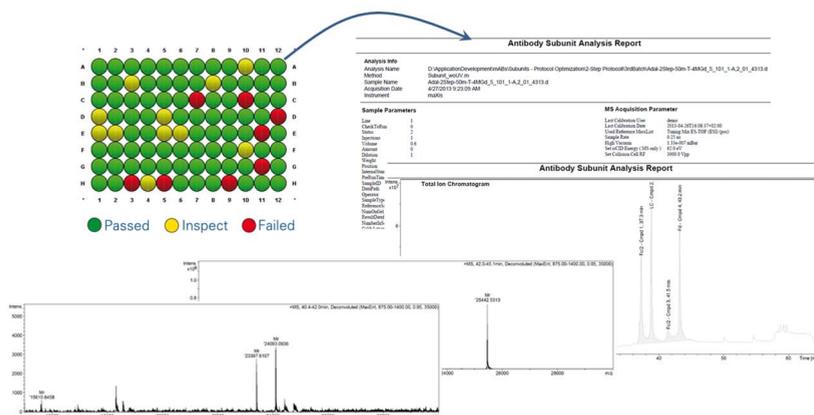
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Figure 16. Automated report of BioPharmaCompass containing essential QC information such as TIC, deconvoluted protein mass and qualitative and quantitative comparison with a reference standard. Incorrect products and impurities are indicated using traffic-light colors for at-a-glance QC.

Example 2: PSA

The following results were generated during the 2013 gPRG study of the Glycoprotein Research Group (gPRG) of the Association of Biomolecular Resource Facilities (ABRF). The main objective of the study was the quantitation of the glycosylation heterogeneity present in two different preparations of human prostate-specific antigen (PSA), which is a biological biomarker for prostate cancer. A poster presented at the 2013 meeting of the ASMS focused on the identification of a new *N*-glycosylation site in the PSA study sample [12]. In brief, the glycosylation profiles of two PSA samples were analysed in a two-way approach: analysis of a tryptic digest by RP HPLC coupled to an amaZon Speed ion trap (Bruker) with CID and ETD capability (“bottom-up”), and analysis of the intact glycoprotein by RP HPLC coupled to a maXis 4G ESI-UHR-TOF (Bruker).

Because ion trap CID spectra of *N*-glycopeptides mainly contain peaks from glycan fragments (B- and Y-type ions) and peptide fragment peaks are rarely seen, little information about the peptide backbone is obtained. Therefore, both CID and ETD spectra were acquired. In contrast to CID, ETD cleaves the *N*-C α bond of the peptide backbone, resulting in c- and z-ion series. Post-translational modifications – for example glycans – remain attached to the respective amino acid residues. An example is shown in Figure 17. More information on ETD of glycopeptides can be found in [13]. As a result, 50 glycan compositions could be assigned to the glycan profile with quantitative information. From 44 glycans, the structure could be deduced from “bottom-up” CID spectra (Figure 18).

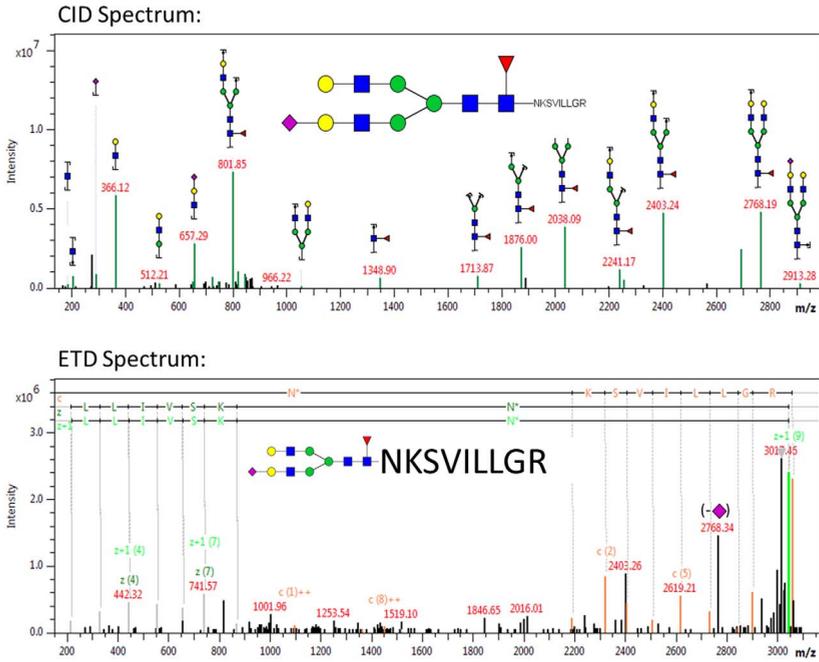


Figure 17. CID and ETD spectrum of a single glycopeptide derived from PSA. The composition of the glycan moiety can be identified by submission of the CID spectrum to GlycoQuest. The peptide moiety can be identified by submission of the ETD spectrum to Mascot once the glycan composition is determined.

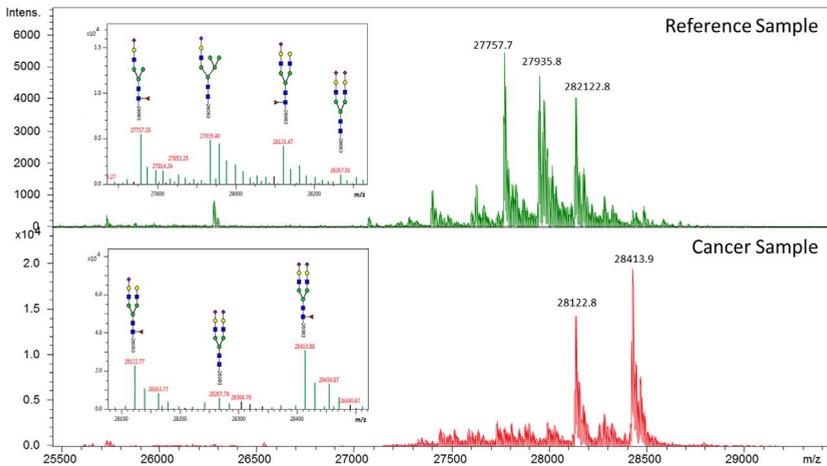


Figure 18. Intact mass spectra of the two PSA samples, annotated with the glycan structures identified from the “bottom-up” approach.

MS of intact glycoproteins is highly suited to the quantitative assessment of glycoprofiles. Because the glycans carry a huge protein residue, suppression effects familiar from complex glycan or glycopeptide spectra, are eliminated [14]. In addition, this approach avoids artefacts that can arise from differences in charge-state distributions or incomplete or unspecific proteolytic digestion. However, only glycoproteins with a single glycosylation site can be quantified without further fragmentation of the protein.

CONCLUSION

The characterisation of glycoproteins is a complex task. In addition to the complete assignment of the amino-acid sequence and the analysis of other post-translational modifications (e.g., phosphorylation) and laboratory artefacts (e.g., oxidation), the investigation of glycosylation and its protein-specific heterogeneity adds further analytical challenges. Because many proteins contain several potential glycosylation sites, and due to a high degree of glycan heterogeneity at a given amino acid, glycosylation patterns are often very complex. Three strategies for the analysis of glycoproteins have been described here:

1. Analysis of released glycans: Reduced complexity, lower mass range, but only very limited information about the glycosylation site(s). Porous graphitised carbon chromatography can separate isobaric glycans.
2. Analysis of glycopeptides in a proteolytic digest: The link to the glycosylation site is retained. However, glycoprotein digests are complex mixtures, and in many cases a specific glycopeptide enrichment step is necessary.
3. Analysis of the intact glycoprotein: Yields the exact quantitative glycosylation profile. Not suitable for proteins with several glycosylation sites.

In many cases a complete overview of the protein's actual state of glycosylation can be produced only by the combination of two or even all three strategies. We have introduced dedicated bioinformatics software that supports each single strategy and significantly facilitates a combined approach.

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