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WHITEPAPER

HIGHLY SENSITIVE ANALYSIS OF GLYCANS IN THERAPEUTIC GLYCOPROTEINS

...With HPAEC-PAD

Quality control during the manufacturing process of therapeutic glycoproteins requires suitable analytical methods to monitor consistency and stability of the critical quality attributes such as the glycome. High-Performance Anion Exchange Chromatography (HPAEC) coupled to Pulsed Amperometric Detection (PAD) is a highly sensitive and selective technique for the analysis of intact glycans as well as the monosaccharides building blocks. Even minor variations in the carbohydrate structures can be detected quickly making the method an essential tool during early drug development, process monitoring and validation as well as batch release testing during routine analysis.

INTRODUCTION

On the biopharmaceutical market, therapeutic proteins have gained increasing significance over the past years and are nowadays applied for the treatment of a wide range of pathologies and diseases. Most of the naturally occurring proteins contain carbohydrate chains covalently linked to the amino acid backbone constituting between 1 to 90% of proteins molecular weight, therefore termed glycoprotein. The class of therapeutic glycoproteins covers monoclonal antibodies (mAbs), hormones, cytokines, enzymes and growth factors amongst others. Erythropoietin as the most prominent example is a highly glycosylated protein, which is used as a therapeutic agent to treat anemia arising from chronic kidney disease, inflammatory bowel disease or chemotherapy. It is one of the two first approved glycoprotein therapeutics. Naturally produced by the kidney, the cytokine stimulates the production of red blood cells in the bone marrow [1]-[3].

The glycosylation of a protein is a key player in many biological processes. Carbohydrates are not only involved in the proper folding of the polypeptide chain in a protein, but also responsible for its stability, solubility, trafficking and various signaling processes (cell-cell and cell-matrix interactions). In addition, glycans take part in pathological processes such as inflammation, immune system activation, bacterial adhesion and viral infection by molecular recognition. Therefore, gaining more knowledge about the complex correlation between glycan structure and function is of great importance. In case of therapeutics, pharmaco-kinetics, pharmacodynamics as well as efficacy of the drug may be affected by the glycosylation pattern, wherefore a therapeutic protein's glycome needs to be carefully controlled [2][3].

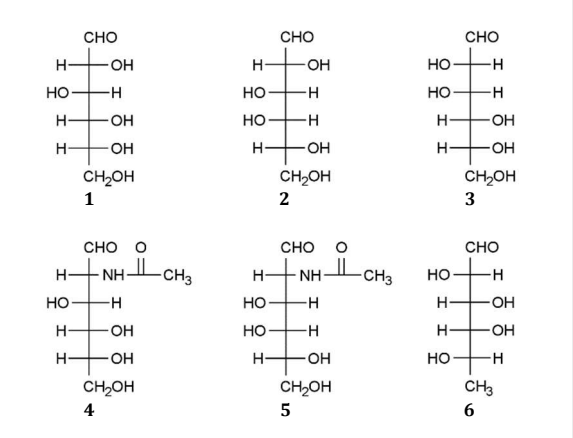

Fig. 1: The most common monosaccharides in human glycoproteins shown in the open-chain Fisher projection: **1**: D-Glucose (Glc), **2**: D-Galactose (Gal), **3**: D-Mannose (Man), **4**: *N*-Acetyl-glucosamine (GlcNAc), **5**: *N*-Acetyl-galactosamine (GalNAc), **6**: L-Fucose (Fuc).

In contrast to the amino acid backbone of a protein, which is encoded in the DNA sequence, the glycome is produced in a non-templated manner in the endoplasmatic reticulum (ER) and Golgi apparatus. Glycosylation is the most common post-translational modification (PMT) of a protein. Enzymes such as glycosylases, glycosyltransferases and sulfotransferases built up the carbohydrate structures from the monosaccharide building blocks in a coordinated action [4][5]. Most common monosaccharides occurring in human glycoproteins are shown in Fig. 1.

In addition to the neutrally charged monosaccharides at physiological pH (following termed "neutral monosaccharides/carbohydrates"), sialic acids are often found to terminate the carbohydrate chains. Sialic acids as derivates of neuraminic acid are more acidic than other monosaccharides and therefore contribute to the

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solubility of a protein. *N*-Acetylneuraminic acid (Neu5Ac, NANA) is the most common member (see Fig. 2) of a large family containing more than 50 derivates [6].

Within oligo- or polysaccharides, the monosaccharides are linked through glycosidic bonds, which are products of condensation reactions between the hemiacetal and the hydroxyl group of two sugar units.

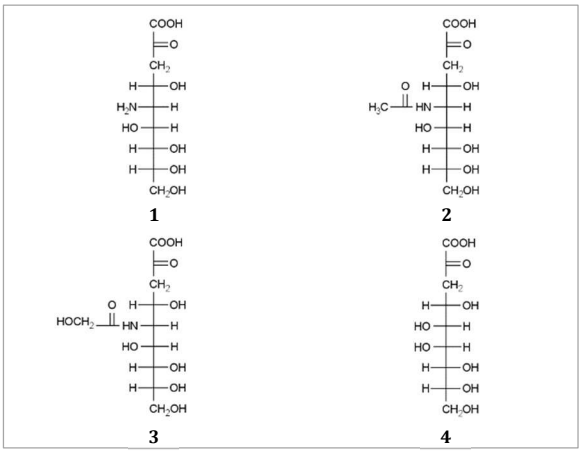

Fig. 2: Four sialic acids shown in the open-chain Fisher projection. **1**: Neuraminic acid (Neu) and its main derivatives: **2**: *N*-Acetylneuraminic acid (Neu5Ac, NANA), **3**: *N*-Glycolylneuraminic acid (Neu5Gc, NGNA), **4**: 2-Keto-3-deoxynonic acid (Kdn).

Even though glycans are built up from a limited number of monosaccharides, an enormous amount of possible structural variants arises. Oligosaccharides can be linked in varying degrees to different sites of the amino acid backbone and show different branching and linking (via 2, 3, 4 or 6 position of hexose and α- or β- conformation of monosaccharide). Glycoforms are proteins with the same amino acid backbone but varying glycosylation patterns [2].

Carbohydrate chains can be linked to amino acid residues via nitrogen and oxygen atoms, thus termed *N*- and *O*-glycans. Starting point of carbohydrate structures in *N*-glycans is *N*-acetyl-glucosamine (GlcNAc) attached to the asparagine within an asparagine – X-serine/threonine motif (X: any amino acid except proline). Three main structural types (see Fig. 3) are known: the oligomannose, complex and hybrid type. All these structures have a $Man_3GlcNAc_2$ core in common. On this basis, oligomannose type glycans contain two additional GlcNAc units, a variable number of mannose (Man) and sometimes glucose (Glc) residues. Complex type glycans contain additionally a variable number of GlcNAc, galactose (Gal), fucose (Fuc), *N*-Acetyl-neuraminic acid (Neu5Ac) and sometimes *N*-Acetyl-galactosamine (GalNAc) residues. Hybrid type glycans combine features of both types. For *O*-glycans no consensus sequence is known. The carbohydrate structures are bound to a serine or threonine residue through GalNAc. These GalNAc linked glycans are known as mucin-type *O*-glycans containing seven basic core structures [7].

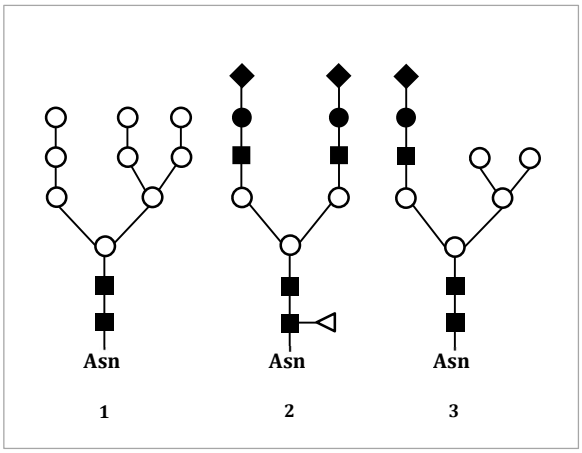

Fig. 3: Three general types of *N*-Glycans, **1**: Oligomannose type, **2**: Complex type, **3**: Hybrid type. ○ is mannose (Man); ● is galactose (Gal); ■ is *N*-Acetyl-Glucosamine (GlcNAc); △ is fucose (Fuc); ◆ is *N*-Acetylneuraminic acid (Neu5Ac).

Glycosylation highly dependent on the expression system

Since different organisms contain different glycosylation machineries, the glycome is highly dependent on the expression system. *N*-Glycolylneuraminic acid (Neu5Gc, NGNA) for example is normally not occurring in human cells except in certain tumor cell lines, but commonly produced in other mammalian cell lines. Injection of therapeutics containing Neu5Gc can lead to an immunogenic reaction due to anti-Neu5Gc antibodies present in human tissue. Therefore, special attention needs to be paid to the compatibility of glycoprotein therapeutics with human hosts to avoid any potential immunogenic response. In addition, proper glycosylation improves the properties of a recombinant

protein by increasing its stability and half-life in blood circulation. Especially, terminal sialic acids have a significant influence on the maintenance of the protein in the bloodstream. Optimal sialylation of a therapeutic glycoprotein can therefore reduce the frequency of injection or needed concentration of a protein in a single dose [6][8][9]. Glycosylation patterns as close as possible to human cells are desirable and can be approached by the choice of a proper expression system. Therefore, most of the today approved therapeutics are expressed in mammalian cell lines such as Chinese Hamster Ovary (CHO) and baby hamster kidney (BHK-21) cells. However, the production of large quantities of recombinant glycoproteins is most promising in yeast, insect and plant cells, which do not express sialic acids and show different glycosylation pattern to humans. To circumvent this defect, genes encoding terminal human glycosyltransferase and other appropriate enzymes can be transfected to the expression cells enabling the production of specific glycoforms (glycosylation engineering) [2][3][6].

To ensure product efficacy and safety due to possible immune reactions as well as proper pharmacokinetics and pharmacodynamics, glycosylation needs to be controlled during the whole life cycle of a therapeutic glycoprotein by utilizing the proper analytical tools.

Analysis of glycans

Roughly speaking, analytical methods can be divided in two categories: methods for in-depth analysis of the carbohydrate structures and methods, which are needed to demonstrate batch-to-batch consistency of a protein's glycosylation. Glycans can be analyzed while being attached to the protein (peptide mapping, analysis of glycopeptides), after intact release from the protein (oligosaccharide profiling) or after being hydrolyzed to the single monosaccharides (monosaccharide analysis) (see Tab. 1). During the drug life cycle, varying analysis degrees of the glycosylation pattern are needed [2].

Early drug development often requires the exact elucidation of the oligosaccharide units in terms of composition, linkage and three-dimensional structure in order to understand their function within the glycoprotein. Different methods need to be combined to get the full picture due to the similar and complex structures of the carbohydrates. Later during the manufacturing process, emphasis is mainly on batch-to-batch consistency, which is also of major concern for regulatory agencies rather than identifying each oligosaccharide on the protein. Fast availability of results to compare the glycosylation of many samples and simplicity in operation is of outmost importance. Commonly used analytics include monosaccharide and oligosaccharide profiling as well as the quantification of sialic acids, which are essential for the half-life of a therapeutic agent [2][6].

Most commonly the carbohydrates are first separated from other interfering species contained in the matrix by application of electrophoretic (CE, PAGE, IEF) or chromatographic methods (RP/IEX-HPLC, HILIC, HPAEC). Detection of carbohydrates after the separation is not trivial due to the absence of a chromophore or fluorescent moiety. Derivatization kids can provide remedy but are expensive and laborious. On the other hand, making carbohydrates accessible to conductivity detection requires very basic environments, but still low responses are obtained. Most common detection methods are mass spectrometry providing structural information, pulsed amperometric detection (PAD) and laser induced fluorescence (LIF) requiring glycan derivatization.

Analyte	Method
Intact glycoproteins	CE, lectin microarray, MS (MALDI or ESI), PAGE/IEF, RP/IEX-HPLC
Glycopeptides	LC-ESI/MALDI-MS, CE-MS
Released glycans, derivatized	MALDI-TOF MS, HPLC (HILIC, RP, AE), LC-FLR, LC-ESI-MS, CE-LIF
Released glycans, underivatized	MALDI-MS, HPAEC-PAD
Monosaccharides, derivatized	RP HPLC-FLR, CE-LIF, GC-MS
Monosaccharides, underivatized	HPAEC-PAD

Tab. 1: Possible methods for the different stages of glycan analysis [1].

HPAEC-PAD for carbohydrate analysis

A highly advantageous approach

HPAEC-PAD is a powerful technique for the analysis of mono-, oligos- and even polysaccharides and is well established for batch release testing during routine analysis of therapeutic glycoproteins. The carbohydrates are separated in an alkaline eluent on an anion exchange column according to their acidity. Subsequently the analytes are detected by amperometry.

With pulsed amperometric detection (PAD) only electroactive species are recorded, which makes the detector highly selective towards reducing carbohydrates even in complex matrices. Due to an excellent S/N ratio, detection limits (LOD) down to pico- or even femtomole range can be reached even though the substance conversion is only around 10%. Competing detection techniques such as refractive index (RI) or absorbance at low UV wavelength do not come close to the required sensitivity or selectivity for the determination of monosaccharides. Compared to fluorescence detection, less sample preparation is needed with PAD as a direct detection method, which avoids analyte derivatization. HPAEC-PAD is inexpensive in contrast to mass spectrometry and simple to operate for an analyst with limited knowledge in carbohydrate chemistry. The resulting methods are robust providing excellent accuracies and precisions and have a broad working range (at least 4 orders of magnitude), which makes them suitable to be validated according to the current analytical requirements [10][11].

The information content, which can be gained from the measurement is high covering the total glycan content, free glycan content and monosaccharide composition. In addition, hints about glycan type can be obtained by calculation of the monosaccharide ratios, the average glycan length and the glycan/protein ratio.

Concluding, HPAEC-PAD is the method of choice for routine analysis of carbohydrates within therapeutic glycoproteins [11].

Sample preparation

Depending on the target analytes, different sample preparation steps are necessary prior to the analysis. It is common to use an internal standard in order to balance for smaller inaccuracies during the sample handling procedure.

For the analysis of glycans (oligosaccharide profiling), the intact oligosaccharides are released from the protein. Either chemical conditions (for *N*-glycans: hydrazine, *O*-glycans, hydrazine or β-elimination ($NaBH_4$ in 0.1 M NaOH, 45 °C, 16–20 h)) or the corresponding glycosidase enzymes can be used in this context.

If the monosaccharides are of interest, the glycans need be split up into the single building blocks by using harsher conditions. Hydrolysis is usually carried out in a strong acidic environment at elevated temperatures. As schematically displayed in Fig. 5, different workflow paths are necessary for the quantification of either the total glycan content or the free glycan content. Determination of the free glycan content is a measure for the degradation of a therapeutic glycoprotein and of particular interest during stability studies in the drug development process. For the quantitative analysis, in a first step the glycoconjugate is removed either by chromatography using a suitable cartridge or molecular weight cut off (MWCO) filters, where the glycoconjugate is retained by a porous membrane. For analysis of the total glycan content, different hydrolysis conditions are required to get either the neutral monosaccharides or the sialic acids.

The choice of hydrolysis conditions is always a balance between release and destruction of the carbohydrates. Although hydrolysis conditions need to be fine-tuned for each glycoprotein, the following conditions can be seen as a guideline [11].

For release of the sialic acids, milder conditions (0.1 M TFA, 1 h, 100 °C) are necessary leaving the proteins with the neutral monosaccharides mostly intact. For

hydrolyzation of the neutral monosaccharides much harsher conditions (2 M TFA, 4 h, 100 °C) are needed, which lead to the destruction of the sialic acids.

It must be considered that although amino sugars (GlcNAc, GalNAc) are usually treated the same way as other neutral carbohydrates, less yield is obtained (only 90–95%) and the amino groups get deacetylated resulting in *N*-Glucosamine (GlcN) and *N*-Galactosamine (GalN). For complete release of amino sugars, 6 M HCl needs to be applied for 4 h at 100 °C, which degrades the other neutral monosaccharides.

It is common to treat the monosaccharide standards under the same hydrolysis conditions as the protein sample to compensate for the slight destruction of the sugars. However, the monosaccharides within the standards will be degraded earlier than the sample monosaccharides, which are first released from the glycoprotein. Therefore, a slight overcorrection of the monosaccharide destruction is inevitable.

Separations with HPAEC

Due to their strong hydrophilicity, it is not possible to separate carbohydrates applying conventional chromatographic methods (HPLC). Anion exchange chromatography on the other hand requires charged species to be retained by the basic resin. Since many carbohydrates have high dissociation constants (around or above 12), they need to be deprotonated first at high pH enabling a separation as oxyanions. As a drawback, epimerization of sugars is fast in very alkaline medium, wherefore anomeric forms can no longer be resolved.

Due to missing stability of anion exchange resins in highly alkaline environments for a long time, only charged (sialylated, phosphorylated or sulfated) glycans could be analyzed with conventional anion exchange chromatography. Only with development of improved column packings in the 1980s, neutral carbohydrates could be separated. Today, columns of the family of Thermo Scientific Dionex CarboPac™ are frequently used for this application. The resins are based on nonporous pellicular particles (6.5–10 µm particle size) made of polystyrene crosslinked with divinylbenzene to different degrees (2–55%). The particles are agglomerated with a microbead latex functionalized with quaternary ammonium ions. The usage of uniform and nonporous particles is advantageous due to a rapid mass transfer during the interaction of the carbohydrates with the functional groups at their surface, which minimizes peak broadening. Quaternary ammonium functional groups at the surface of the particles enable the separation of the monosaccharides according to their pK_a value. Mannose as the most acidic of the carbohydrates displayed in Fig. 1 for example will elute last (see Fig. 8). Since the processed protein samples may contain species interfering with the detection of carbohydrates, it is frequent practice to add a trap column in between injector and separation column. An amino acid trap column retains small peptides and amino acids, which otherwise coelute with the carbohydrates. Especially for lightly glycosylated proteins (e. g. monoclonal antibodies) this is a major issue. Carbonate, borate and other interfering anions can be removed by using an anion or borate trap column [11]-[13].

The chromatographic methods usually contain an eluent gradient to remove species strongly bound to the resin and afterwards re-equilibrate the column. Special attention must be paid to the possible accumulation of carbonate over time because alkaline solutions strongly adsorb carbon dioxide from the atmosphere. As a result, column capacity may be decreased as well as selectivity, resolution, efficiency and reproducibility. Therefore, it is important to degas the eluents prior to use [11].

Redox chemistry in amperometry

Amperometric detection is based on the redox reaction of the analyte on an electrode surface by application of a potential and measurement of the resulting current over time. The electric current arising is directly proportional to the concentration of analyte oxidized or reduced, which is described by Faraday's law.

I_t is the arising current at time t, Q is the charge at the working electrode, n is the number of electrons transferred per mole of analyte, F is the Faraday constant (96485 C mol^{-1}), and N is the number of moles oxidized or reduced. After measuring the current over a defined time length, charge (Q) is obtained with the detector response measured in Coulomb (C).

$$I_t = \frac{dQ}{dt} = nF\frac{dN}{dt} \qquad [1]$$

A significant drawback when applying a constant potential in amperometric detection, is the fouling of the electrode surface due to accumulation of interfering species during the redox reaction. Therefore, usually a pulsed (PAD: pulsed amperometric detection) rather than a constant potential is applied to keep the electrode reactive and clean, which leads to a drastic improvement of the analytical reproducibility. In carbohydrate analysis most often a four-step potential waveform (QPAD: quadrupole potential amperometric detection) is used (see Fig. 7). The detection of the analyte during time t_{det} follows an initial time t_{del}, which is needed for the charging current to decay. The charging current arises due to the formation of an electric double layer after switching on the potential. During the detection time t_{det}, the current resulting from the oxidation of the carbohydrates at the gold electrode is measured. While the aldehyde functionality of the carbohydrate is catalytically oxidized to the corresponding carboxyl group yielding a sugar acid in a first reaction step (see Fig. 4), gold (III) hydroxide ($Au(OH)_3$) species get reduced. The transfer of an electron from the absorbed carbohydrate to the electrode is the rate determining step of this kinetically limited reaction. Therefore, the flow geometry of the carrier stream has a large influence on the detector performance. The formation of higher gold oxides (Au_2O_3) species causes the catalytic oxidation to stop. Therefore, a surface cleaning step follows the detection. During time t_{red} a large negative potential is applied to reduce solvated gold oxide species back to solid gold. With a brief positive potential in the following step during time t_{oxd}, the electrode surface is reactivated by formation of catalytic gold (III) hydroxide ($Au(OH)_3$). The last step during time t_{ads} consists of applying a negative potential to disrupt the adsorption of the analyte on the electrode surface [14]-[16].

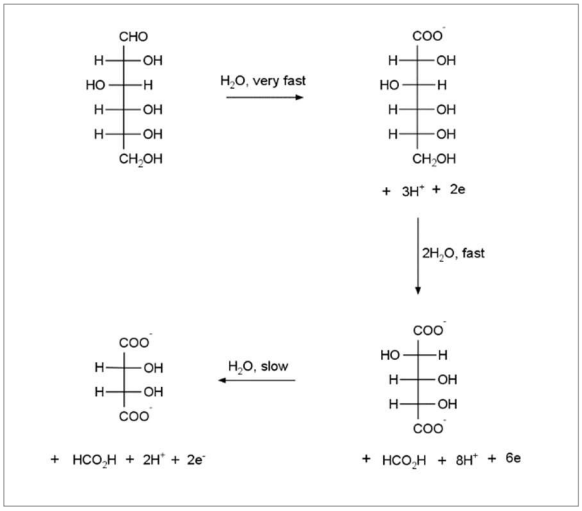

Fig. 4: Oxidation reactions of the analyte at the electrode surface, exemplarily shown on glucose [16].

The PAD consists of a small flow cell containing three electrodes (see Fig. 6). The working potential is applied between working and auxiliary electrode, which is the detector block itself made of stainless steel. At the working electrode the actual redox reaction takes place. The electrode is usually made of noble metals such as gold, silver, platin but also carbon electrodes are available for special applications. The resulting current is recorded over time using an amperemeter. The reference electrode, which is often a silver chloride electrode (Ag/AgCl) ensures that the correct predefined potential is applied to the working electrode.

Conclusion

HPAEC-PAD is the state-of-the-art analysis technique for the quantification of carbohydrates in therapeutic proteins. Excellent sensitivity, good selectivity, simple handling and high robustness characterize the technique. Methods can be easily validated according to the current analytical requirements and afterwards applied for batch release testing during routine manufacturing.

Sample preparation protocol for monosaccharide analysis

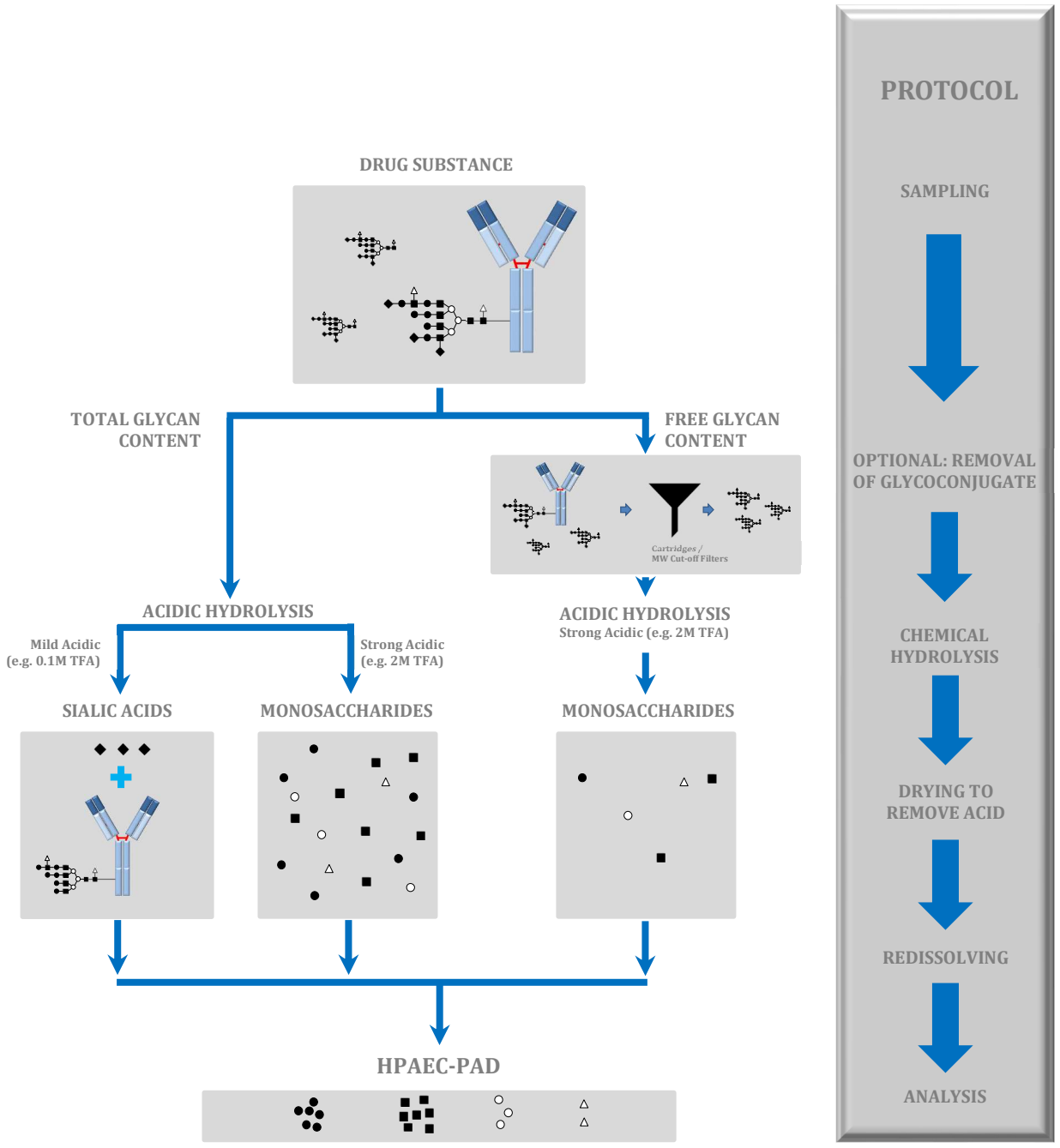

Fig. 5: Scheme representing the workflow for sample preparation methods for the analysis of different monosaccharide types. Either total glycan content can be determined by acidic hydrolysis of the glycoconjugate. Mild hydrolysis conditions lead to the removal of sialic acids and intact glycoconjugate while harsh hydrolysis conditions lead to destruction of the sialic acids and the glycoconjugate to yield the only the neutral monosaccharides. To quantify the free glycans fallen off during storing, either cartridges or molecular weight cut-off filters need to be applied to remove the glycoconjugate.

Measuring Principle

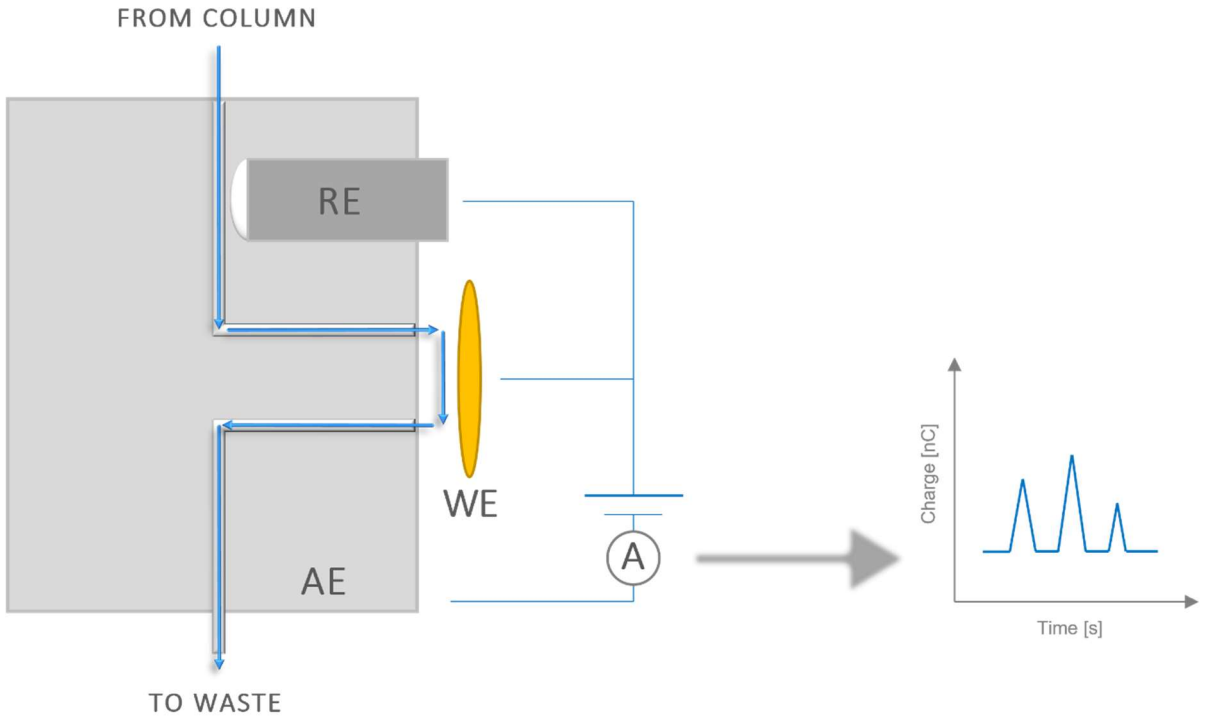

Fig. 6: Amperometric measuring principle: Effluent from the column enters the detector flow cell on top, reference electrode (RE) delivers the reference potential for the working electrode (WE), where the redox reaction takes place. The potential is applied between working and auxiliary electrode (AE). Charge is measured by an amperemeter in dependency of time, which can be plotted in a chromatogram when coupled to chromatography.

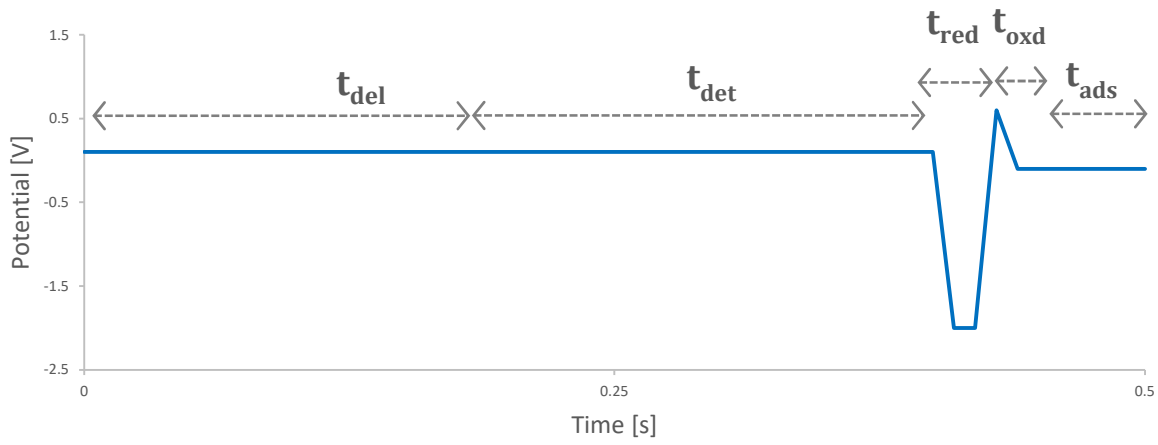

Fig. 7: Pulse regime in PAD. Quadrupole-potential waveform (QPAD) consisting of four different potentials applied during five time steps. t_{del}: delay time required for the charging current to decay; t_{det}: recording of current resulting from the oxidation reaction; t_{red}: reduction of electrode surface; t_{oxd}: reactivation of electrode surface; t_{ads}: negative potential to avoid absorption of the analyte.

Example chromatograms

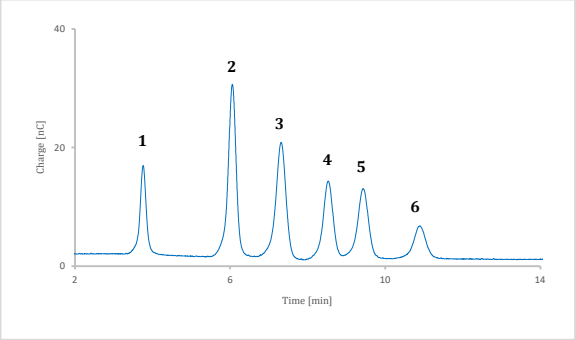

Fig. 8: Chromatogram showing the separation of six of the most common monosaccharides contained in human glycoproteins at a concentration of 50 nmol/L. **1**: L-Fuc, **2**: D-GalN, **3**: D-GlcN, **4**: D-Gal, **5**: D-Glc, **6**: D-Man.

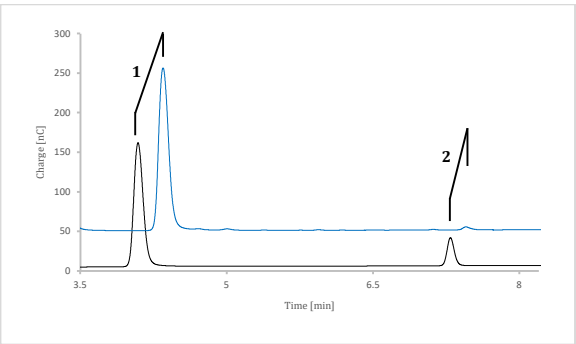

Fig. 9: Chromatograms demonstrating the separation of sialic acid derivates. **1**: Neu5Ac and **2**: Neu5Gc. Standard solution containing Neu5Ac and Neu5Gc at 20 µg/mL respectively 1.5 µg/mL (black) and protein sample (blue) after acidic hydrolysis (0.1 M HCl, 1 h, 80 °C).

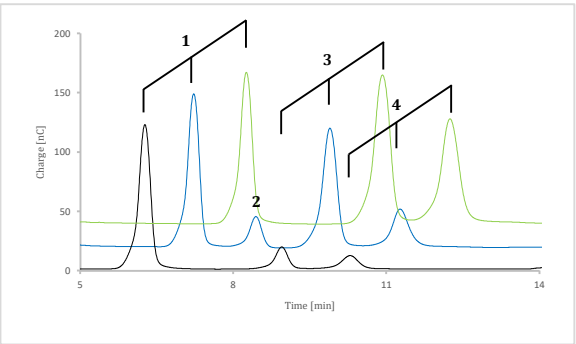

Fig. 10: Chromatogram showing a method for the quantification of the total glycan content of a therapeutic protein after hydrolysis (2 M TFA, 4 h). **1**: GalN as an internal standard, **2**: GlcN, quantification of **3**: Glc and **4**: Man. Standard solution containing the analytes in 2.5 µg/mL (black), protein sample (blue), check standard solution in 30 µg/mL (green).

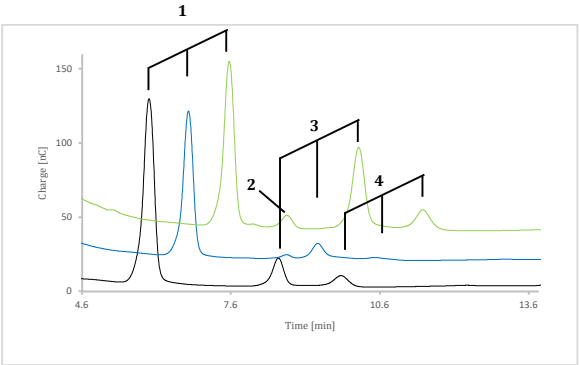

Fig. 11: Chromatogram showing a method for the quantification of the free glycan content in a drug product after removal of the glycoconjugate, hydrolysis (2M TFA, 4h). **1**: GalN as internal standard, **2**: GlcN, quantification of **3**: Glc and **4**: Man. Standard solution containing the analytes at 2.5 µg/mL (black), protein sample (blue), spiked protein sample (green).

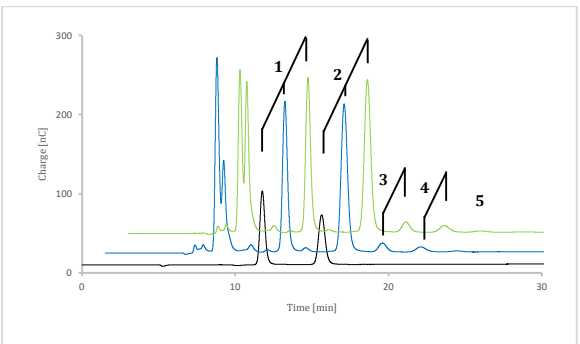

Fig. 12: Quantification of mannose from *O*-linked glycans in a monoclonal antibody by *β*-elimination under alkaline conditions ($NaBH_4$ in 0.1 M NaOH, 45 °C, 16 – 20 h). **1**: Arabitol, **2**: Mono-mannitol, **3:** Di-mannitol, **4**: Tri-mannitol, **5**: Tetra-mannitol. Standard solution (black), test sample (blue) and *O*-glyco check solution (green).

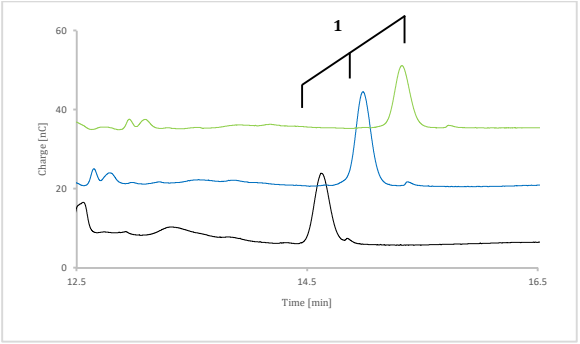

Fig. 13: Quantification of the uronic acid **1**: *N*-Acetyltalosaminuronic acid (AltNAcA) in a bioconjugate after acidic release contained in the standard solution at 40 µg/mL (black), protein sample (blue), check standard solution (green).

List of acronyms

AltNAcA	*N*-Acetyltalosaminuronic acid
BHK-21	Baby hamster kidney
CE	Capillary electrophoresis
CHO	Chinese hamster ovary
ER	Endoplasmatic Reticulum
ESI	Electrospray ionization
LIF	Laser induced fluorescence
FLR	Fluorescence
Fuc	Fucose
Gal	Galactose
GalN	*N*-Galactosamine
GalNAc	*N*-Acetyl-galactosamine
GC	Gas chromatography
Glc	Glucose
GlcN	*N*-Glucosamine
GlcNAc	*N*-Acetyl-glucosamine
HILIC	Hydrophilic interaction chromatography
HPAEC	High-performance anion exchange chromatography
HPLC	High-performance liquid chromatography
IEF	Isoelectric focusing
IEX	Ion exchange
Kdn	2-Keto-3-deoxynonic acid
LOD	Limit of detection
mAbs	Monoclonal antibody
MALDI	Matrix assisted laser desorption ionization
Man	Mannose
MS	Mass spectrometry
MWCO	Molecular weight cut-off
Neu	Neuraminic acid
Neu5Ac, NANA	*N*-Acetylneuraminic acid
Neu5Gc, NGNA	*N*-Glycolylneuraminic acid
PAD	Pulsed amperometric detection
PAGE	Polyacrylamide gel electrophoresis
PMT	Post-translational modification
RI	Refractive index
RP	Reversed phase
S/N	Signal-to-noise ratio
TOF	Time of flight

References

[1] Zhang, L., Luo, S., Zhang, B. Glycan analysis of therapeutic glycoproteins. *MABS* **2016**, *8*, 205−215.

[2] Higgins, E. Carbohydrate analysis throughout the development of a protein therapeutic. *Glycoconj. J.* **2010**, *27*, 211−225.

[3] Valverde, P., Ardá, A., Reichardt, N.-C., Jiménez-Barbero, J., Gimeno, A. Glycans in drug discovery. *Med. Chem. Commun.* **2019**, *10*, 1678.

[4] Geyer, H., Geyer, R. Strategies for analysis of glycoprotein glycosylation. *Biochimica et Biophysica Acta.* **2006**, *1764*, 1853−1869.

[5] Hevey, R. Strategies for the development of glycomimetic drug candidates. *Pharmaceuticals* **2019**, *12*, 55.

[6] Varki, A., Schauer, R. Essentials of Glycobiology; Cold Spring Harbor Laboratory Press, 2009.

[7] Bagdonaite, I. et al. Glycoproteomics *Nature Reviews* **2022**, *2*:48.

[8] Raju, T.S.: Glycosylation variations with expression systems and their impact on biological activity of therapeutic immunoglobulins. *Bioprocess Int* **2003**, 44–54.

[9] Lalonde, M.-E., Durocher, Y. Therapeutic glycoprotein production in mammalian cells. *Journal of Biotechnology* **2017**, *251*, 128−140.

[10] ICH Guideline Q2 (R1), "Validation of analytical procedures: Text and methodology".

[11] Bhattacharyya, L.; Rohrer, J. S. Applications of Ion Chromatography for Pharmaceutical and Biological Products; John Wiley & Sons, 2012.

[12] Thermo Scientific, "Dionex CarboPac PA1", Column Product Manual, 2013.

[13] Thermo Scientific, "Dionex CarboPac PA20-Fast-4µm", Column Product Manual, 2017.

[14] LaCourse, W. R. Pulsed electrochemical detection in high-performance liquid chromatography. John Wiley & Sons: 1997.

[15] LaCourse, W. R., Johnson, D. C. Optimization of waveforms for pulsed amperometric detection (PAD) of carbohydrates following separation by liquid chromatography, *Carbohydr. Res.* **1991**, *215*, 159-178.

[16] Johnson, D. C., LaCourse, W. R. Liquid chromatography with pulsed electrochemical detection at gold and platinum electrodes, *Anal. Chem.* **1990**, *62*, 589-597.