Monitoring of immunoglobulin N- and O-glycosylation in health and disease

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Abstract

Protein N- and O-glycosylation are well known co- and post-translational modifications of immunoglobulins. Antibody glycosylation on the Fab and Fc portion is known to influence antigen binding and effector functions, respectively. To study associations between antibody glycosylation profiles and (patho) physiological states as well as antibody functionality, advanced technologies and methods are required. In-depth structural characterization of antibody glycosylation usually relies on the separation and tandem mass spectrometric (MS) analysis of released glycans. Protein- and site-specific information, on the other hand, may be obtained by the MS analysis of glycopeptides. With the development of high-resolution mass spectrometers, antibody glycosylation analysis at the intact or middle-up level has gained more interest, providing an integrated view of different post-translational modifications (including glycosylation). Alongside the in-depth methods, there is also great interest in robust, high-throughput techniques for routine glycosylation profiling in biopharma and clinical laboratories. With an emphasis on IgG Fc glycosylation, several highly robust separation-based techniques are employed for this purpose. In this review, we describe recent advances in MS methods, separation techniques and orthogonal approaches for the characterization of immunoglobulin glycosylation in different settings. We put emphasis on the current status and expected developments of antibody glycosylation analysis in biomedical, biopharmaceutical and clinical research.

Key words: antibody, biopharmaceutical, glycan, glycoproteomics, mass spectrometry

Introduction

The diverse roles of glycosylation in various biological and pathophysiological processes as well as the importance of protein glycosylation in the development of biopharmaceuticals have, over the past decade, received broad appreciation in the life sciences (Walt et al. 2012). The best studied glycoproteins in terms of the structure and function of their glycosylation are immunoglobulins (Figure 1), in particular human immunoglobulin G (IgG; Arnold et al. 2007; Dekkers et al. 2017) which features mostly complex biantennary glycans with varying degrees of galactosylation, sialylation, bisection and core fucosylation. Since the 1980s a vast body of literature has become available, detailing immunoglobulin glycosylation features across the different antibody isotypes. Additionally, these data increasingly describe immunoglobulin glycosylation in a subclass-, allotype- and site-specific manner (Huhn et al. 2009; Zauner et al. 2013; Plomp et al. 2016). Importantly, antibody glycosylation has been shown repeatedly to differ between sexes and with age as well as with various environmental and life-style factors such as urbanization and smoking (Gudelj et al. 2018). Moreover, antibody glycosylation has been found to be skewed in numerous diseases including various autoimmune diseases, infectious diseases and different types of cancer (Parekh et al. 1985; Ackerman et al. 2013; Gudelj et al. 2018). These
findings mainly concerned IgG glycosylation, but recently IgA glycosylation has also attracted attention and was found to be associated with rheumatoid arthritis as well as pregnancy (Bondt et al. 2017). In accordance with the IgG glycosylation changes observed in health and disease, multiple associations of this antibody glycosylation have been shown with inflammatory markers such as cytokines and C-reactive protein (Plomp et al. 2017). Likewise, associations of IgG glycosylation with markers of metabolic health, such as blood glucose, lipoprotein particles and central adiposity, have been established (Lemmers et al. 2017; Plomp et al. 2017; Russell et al. 2019). Next to development, environment, inflammation and metabolism, a strong genetic and also epigenetic influence on antibody glycosylation has been revealed (Lauc et al. 2010, 2013, 2014; Klasic et al. 2016, 2018).

While our knowledge of human antibody glycosylation has been growing considerably, also the immunoglobulin glycomes of important model systems become increasingly mapped. These include the IgG glycosylation of cell lines, such as CHO, HEK, NS0 and SP2 cells, and various murine strains (Figure 2; Stadlmann et al. 2008; Montesino et al. 2012; Dekkers et al. 2016; de Haan et al. 2017; Goh and Ng 2018) and IgA glycosylation in mammalian expression systems as well as glyco-engineered plants (Dicker et al. 2016; Rouwendal et al. 2016). It is crucial for the proper translation of research results between different systems to take the source-specific glycosylation features of antibodies into account.

Our understanding of the role and diversity of protein glycosylation in general, and antibody glycosylation in particular, has grown in parallel with the development and implementation of new molecular and analytical tools in both biomedical and biopharmaceutical research. The field of glycoengineering has been instrumental for furthering our glycobiological understanding. Prominent developments in this area include (1) molecular biology tools to edit the glycoenzyme makeup of eukaryotic cells, (2) in vitro tools such as small molecule inhibitors and medium additives to modulate protein glycosylation during cell culture and (3) the availability and application of glycosyltransferases and glycosidases for the post-cell culture editing of protein glycosylation (Dekkers et al. 2016; Giddens et al. 2018; Gupta and Shukla 2018). Of equal importance has been the development of analytical tools to aid in the study of both structure and function of specific glycans and glycoforms. Here, released glycan analysis by hydrophilic interaction liquid chromatography with fluorescence detection (HILIC-FLU) has a central role for the analysis of antibody glycosylation in both biomedical and biopharmaceutical settings. HILIC-FLU methods often serve as a gold standard for the characterization of antibody glycosylation, featuring excellent
robustness, accuracy and precision (Reusch, Haberger, Maier, et al. 2015). Although this method provides sufficient resolution to profile the moderately complex global IgG glycome, it does not suffice for the in-depth characterization of site-specific antibody glycosylation in general.

Antibody glycosylation analysis has recently seen several specific developments which are pivotal for further glycobiological research. This includes the development of high-throughput techniques such as capillary gel electrophoresis with laser-induced FLU (CGE-LIF) and various mass spectrometric (MS) approaches (Szekrenyes et al. 2012; Reusch et al. 2014; Reusch, Haberger, Falck, et al. 2015, Reusch, Haberger, Maier, et al. 2015; Szigeti and Guttman 2017). Furthermore, a vast body of work was performed enabling site-specific glycosylation profiling of immunoglobulin isotypes and subclasses analyzing glycopeptides by MS (Selman et al. 2012; de Haan et al. 2015; Bondt et al. 2016; Chandler et al. 2019). Another important aspect of antibody glycosylation analysis is miniaturization in order to increase sensitivity. For example, MS methods have been instrumental for analyzing antigen-specific antibody subpopulations with high sensitivity and specificity (Wuhrer et al. 2009; Kapur et al. 2014).

Analytical technologies that focus on structural features of antibodies have been complemented by methods for studying functional aspects of antibody glycosylation. Of interest here are, for example, the scavenging functions of antibodies and the interactions between antibody Fc portions and receptors involved in effector mechanisms of the humoral immune system. This leads to the integration of structural and function analysis at the intact protein level with important roles for native-mode protein separation and native MS (Gahoul et al. 2017; Kiyoshi et al. 2018).

Here, we provide an overview of recent developments in antibody glycosylation analysis, highlighting advances in separation techniques, mass spectrometric methods and functional assays. We will describe the status, current role and expected development of antibody glycosylation analysis in biomedical research, biopharma and eventually clinical diagnostics.

Separation-based approaches

The limited complexity of Ig glycan structures means separation-based techniques—that is without additional selectivity from MS—often provide sufficient resolution. Due to the limited overlap in glycans between Fc and Fab glycosylation of IgG and the occurrence of solely Fc glycosylation in many monoclonal IgG samples, their glycosylation analysis does, generally, not require a site-specific approach (Bondt et al. 2014). However, it cannot be excluded that this is different under specific (patho) physiological conditions or for Fab glycosylated monoclonal antibodies (mAbs). Therefore, it is advisable to choose a site-specific method in the initial characterization of a (potentially) Fab glycosylated IgG from unfamiliar sources, such as an understudied cell line, biofluid or pathology. Additionally, the other Ig classes have several glycosylation sites in the constant region with overlapping glycan species (Arnold et al. 2007; Plomp et al. 2014, 2018). Hence, glycopeptide analysis by MS-based methods is preferred for these in order to resolve site-specific glycosylation. HILIC-FLU remains the gold standard for released glycan analysis due to its high precision and low implementation hurdle (Reusch, Haberger, Maier, et al. 2015; Colhoun et al. 2018; Table I). While classically 2-aminobenzamide (2-AB) or variants are
### Table I. Overview of techniques used for the analysis of immunoglobulin glycosylation

| Technology                                      | Accessibility | Readiness | Throughput | Glycan isomer resolution | Feature resolution | Sensitivity | Quantitation precision | Site specific | Protein specific |
|------------------------------------------------|---------------|-----------|------------|--------------------------|--------------------|-------------|------------------------|---------------|------------------|
| Separation based                               |               |           |            |                          |                    |             |                        |               |                  |
| HILIC-fluorescence of glycans                  | +             | +         | –          | +                        | –                  | +/-         | +                      | no            | no               |
| CGE-LIF                                        | +             | +         | +          | +                        | –                  | +           | +                      | no            | no               |
| HILIC-fluorescence of glycopeptides            | +             | –         | –          | +                        | –                  | +/-         | +                      | yes           | no               |
| Mass spectrometry based                        |               |           |            |                          |                    |             |                        |               |                  |
| HILIC-LC-ESI-MS of glycans                     | +/-           | +/-       | –          | +                        | +/-               | +/-         | +/-                   | no            | no               |
| CGE-ESI-MS of glycans                          | +             | +         | +          | +                        | +                  | +           | +                      | no            | no               |
| Ion mobility-MS of glycans                     | +/-           | –         | +          | +/-                      | +/-               | +/-         | +/-                   | no            | no               |
| MALDI-MS of glycans                            | +             | +         | +          | –                        | –                  | +           | +                      | yes           | yes              |
| RP-LC-ESI-MS of glycopeptides                  | +             | +         | +/-        | –                        | –                  | +/-         | +                      | yes           | yes              |
| HILIC-LC-ESI-MS of glycopeptides               | +             | +         | –          | +/-                      | +/-               | +/-         | +/-                   | yes           | yes              |
| PGC-LC-ESI-MS of glycans                       | +             | –         | –          | +                        | +                  | +/-         | –                      | yes           | yes              |
| CE-ESI-MS of glycopeptides                     | +/-           | +/-       | –          | +/-                      | +/-               | +/-         | +/-                   | yes           | yes              |
| MALDI-MS of glycopeptides                      | +/-           | +         | +/-        | –                        | +/-               | +/-         | +/-                   | yes           | yes              |
| ESI-MS of intact antibodies                    | +/-           | +         | +/-        | –                        | +/-               | +/-         | +/-                   | no            | yes              |
| ESI-MS of antibody fragments                   | +/-           | +         | +          | –                        | +/-               | +/-         | +/-                   | no (yes for IgG) | yes              |
| MALDI-MS of intact antibodies                  | –             | +/-       | +          | –                        | –                  | +           | +                      | no            | yes              |
| MALDI-MS of antibody fragments                 | –             | +/-       | +          | –                        | –                  | –           | –                      | no (yes for IgG) | yes              |
| RP-LC-ESI-MS of intact antibodies               | +/-           | –         | –          | –                        | –                  | –           | –                      | no            | yes              |
| RP-LC-ESI-MS of antibody fragments             | +/-           | +         | –          | –                        | –                  | –           | –                      | no (yes for IgG) | yes              |
| HILIC-LC-ESI-MS of intact antibodies            | +/-           | +/-       | –          | –                        | –                  | –           | –                      | no            | yes              |
| HILIC-LC-ESI-MS of antibody fragments          | +/-           | +/-       | –          | –                        | –                  | –           | –                      | no (yes for IgG) | yes              |
| CE-ESI-MS of intact antibodies                 | +/-           | +         | –          | –                        | –                  | –           | –                      | no (yes for IgG) | yes              |
| CE-ESI-MS of antibody fragments                | +/-           | +         | –          | –                        | –                  | –           | –                      | no (yes for IgG) | yes              |
| Affinity-LC-ESI-MS of intact antibodies        | –             | +/-       | –          | –                        | +                  | +           | –                      | no            | yes              |
| Other approaches                               |               |           |            |                          |                    |             |                        |               |                  |
| Lectins                                        | +             | +         | +          | +/-                      | –                  | +/-         | +                      | no            | no               |
| Anti-carbohydrate antibodies                   | +             | +         | +          | +/-                      | –                  | +/-         | +                      | no            | no               |
| Affinity-LC-UV of intact antibodies            | –             | +         | –          | +/-                      | +/-               | –           | –                      | no (yes for IgG) | yes              |
| SPR array                                      | –             | +         | –          | +/-                      | +/-               | –           | +/-                   | no            | no               |
| NMR of intact antibodies                       | +/-           | +/-       | –          | +                        | +/-               | –           | +                      | yes           | yes              |

aThe accessibility of the method indicates the hardware availability in an average laboratory.

bThe readiness of the method indicates the ease of implementation of the technology for immunoglobulin glycomics, when the hardware is present.

cPrecision here also takes the robustness of the precision into account.
used for fluorescence labeling (Huffman et al. 2014), procainamide and RapiFluor are becoming increasingly popular due to their improved fluorescence yield (Keser et al. 2018). HILIC-FLU separates based on number of galactoses and N-acetyleneuraminic acids (Neu5Ac) and the absence or presence of core fucose and bisecting N-acetylgalcosamine (GlcNAc). Additionally, galactose can be located to the α1-3 or α1-6 arm, an isomer distinction which is generally achieved with HILIC-FLU and has previously been associated, for example, with systemic lupus erythematosus (Vuckovic et al. 2015). Mixed-mode columns, combining anion exchange (AEX) resins with either HILIC or reversed-phase (RP) stationary phases, have been proposed to resolve co-elution of differently sialylated glycans, similarly to popular weak AEX pre-fractionation (Langy et al. 2017). However, these cases are rare in state-of-the-art HILIC separations of IgG glycans (Keser et al. 2018). HILIC-FLU for glycopeptides, relying on fluorescent labeling of the peptide N-terminus, can be implemented as a simple add-on to well-established peptide mapping workflows (Ludger 2019). Generally, liquid chromatography (LC)-FLU approaches require at least around 30 min per analysis, limiting their throughput.

Alternatively, separations can be performed by capillary electrophoresis (CE)-LIF, using either gel-filled capillaries (CGE) or open capillaries (CZE; Borza et al. 2018; Magorivska et al. 2018; Table I). In most cases, fluorescence labeling is achieved by reductive amination with aminopyrene trisulfonate (APTS), providing three negative charges. CZE capillaries are often coated to prevent a negative influence of wall interactions on peak shape and reproducibility (Magorivska et al. 2018). CE-LIF has a high separation power, resulting in the resolution to separate all N-glycosylation features generally separated by HILIC-LC. This includes the distinction of sialic acid linkage isomers which may be interesting especially in the context of glycoengineering (de Haan et al. 2015). Neutralization of the sialic acids can provide an alternative selectivity between sialylated and non-sialylated glycoforms as demonstrated, for example, for the determination of hypogalactosylation in rheumatoid arthritis (Schwedler and Blanchard 2019). Migration time variation is generally managed by the implementation of standards (Szigeti and Guttman 2017). Fast CZE-LIF analysis times of 3 min have been reported (Szigeti and Guttman 2017). Alternatively, CGE-LIF provides a high-throughput platform via the multiplexing of analysis using modified DNA-analyzers and, more recently, dedicated machines with 12–96 parallel capillaries (Reusch et al. 2014; Sciex 2018). Thus, CE-LIF can be operated at high throughput, although precision seems to be slightly inferior to LC-FLU in these modes (Huffman et al. 2014; Reusch, Habberger, Maier, et al. 2015; Sciex 2018).

**Mass spectrometry-based approaches**

While separation-based methods often suffice for the global analysis of IgG N-glycosylation, the broader and more specific monitoring of Ig glycosylation (including that of IgG) requires MS-based technologies (Table I). These methods have a large advantage over the non-MS-based methods described above, as they add an extra high-resolution dimension, based on mass-over-charge ratio (m/z). They also provide the ability to characterize unknown glycan compositions and structures in complex samples, especially using tandem MS approaches (Reiding et al. 2018). Various complementary MS-based workflows are commonly used which, in combination, provide an in-depth overview of Ig glycosylation. These methods tend to follow one of the following approaches, namely (1) the chemical or enzymatic release of glycans from glycoproteins, (2) the proteolytic cleavage of glycoproteins to obtain a mixture of peptides and glycopeptides or (3) the analysis of the intact glycoproteins.

**Released glycans**

The analysis of released glycans provides the opportunity to perform an in-depth structural characterization of the glycoforms present in a sample, irrespective of the protein they are derived from. Inherent to this approach is the loss of protein- and site-specific information. The latter makes released glycan analysis of IgGs prone to biases introduced by contaminating proteins (Lauc et al. 2018). Additionally, when glycans are released from intact IgGs, no distinction can be made between glycosylation of the constant region, where glycosylation may influence the interaction with effector molecules (Gudel et al. 2018), and the variable region, where glycosylation may impact antigen binding (van de Bovenkamp et al. 2016). On the other hand, the analysis of released glycans seems essential for the characterization of Fab-specific glycosylation of polyclonal antibodies; due to the vast heterogeneity which is often exhibited by polyclonal antibody populations, the proteomic analysis of variable and hypervariable regions is challenging, and consequently Fab glycosylation is currently studied at the released glycan level or using binding assays, rather than site-specific at the glycopeptide level (Bondt et al. 2014; van de Bovenkamp et al. 2018). Fab-specific glycan profiling is facilitated at the released glycan level when the Fab portion is separated from the Fc portion prior to glycan release. This can be obtained by using, for example, the IdeS enzyme that cleaves below the hinge region of IgG (Bondt et al. 2014). The importance of analyzing Fab glycosylation was exemplified in the case of rheumatoid arthritis, where ACPA-IgGs showed high levels of Fab glycosylation as compared to total IgG. These Fab glycosylated ACPA-IgGs may convey specific immunological roles, e.g. via binding to human lectins, and may contribute to the development of IgG-mediated autoimmunity in rheumatoid arthritis (Hafkenscheid et al. 2017).

Furthermore, analysis at the released glycan level is technologically most advanced with regard to the structural elucidation of glycans by hyphenating (tandem) MS to a chromatographic or electrophoretic separation module via electrospray ionization (ESI). Powerful methods for the sensitive structural characterization of glycans in general involve porous graphitized carbon (PGC)-LC-, HILIC-LC-, CE- and ion mobility spectrometry (IMS)-MS (Gennaro et al. 2002; Mauko et al. 2012; Kolarich et al. 2015; Zhong et al. 2015; Harvey et al. 2016; Zhao et al. 2016; Keser et al. 2018). Similar to the separation-based methods, the MS-based analysis of released glycans starts with the purification of the antibody and the chemical or enzymatic release of the N-glycans from the proteins. Subsequently, glycans are reduced, derivatized or kept in their native form, depending on the separation and detection technique used. The choice of the sample preparation and derivatization strategy will have to be aligned with the chromatographic or electrophoretic separation technique as well as with the MS-based analysis method. Negative mode tandem MS is in particular suitable for the in-depth structural characterization of glycan isomers (Everest-Dass et al. 2013; Kolarich et al. 2015; Zhao et al. 2016), while positive mode (tandem) MS is often used for a robust and sensitive profiling (Pabst et al. 2009; Wang et al. 2017). Released glycans are relatively hydrophilic and non-sialylated species often lack readily ionizable groups, two features that hamper their efficient desolvation and ionization by ESI. These limitations are generally addressed by derivatization, e.g. by permethylation or reducing end labeling. Permethylation causes
the methylation of all carboxyl-, hydroxyl- as well as primary and secondary amine-groups of glycans, resulting in their neutralization and an overall higher hydrophobicity (Zhou et al. 2017). However, the permethylation reaction is hard to bring to completion and by-products are often observed. In addition, while permethylation comes with high-sensitivity detection, the sample preparation would need further optimization and downscaling to address limitedly available samples, as the current workflows consume microgram amounts of antibody (Shubhakar et al. 2016; Zhou et al. 2017). Alternatively, the reducing end of the N-glycans is employed for uniform derivatization of all species. This labeling is dependent on either the glycosylamine product that is the direct result of N-glycan release by PNGase F, or the reducing end aldehyde that emerges after hydrolysis of the glycosylamine. Examples of glycosylamine-dependent labels are RapiFluorMS (Lauber et al. 2015) and InstantPC (Kimzey et al. 2015), which are used for the rapid labeling of N-glycans in relatively pure samples. Labels that react with the reducing end aldehyde carry either an amine, aminooxy, hydrazine or hydrazide functional group. For example, the amine labels APTS (Maxwell et al. 2011), 8-aminoazaphenolone-1,3,6-trisulfonic acid (Gennaro et al. 2020), procainamide (Kozak et al. 2015) and 2-AB (Pabst et al. 2009; Zhao et al. 2016) form a Schiff base with the reducing ends of the glycans, that is subsequently stabilized via a reduction step. Alternatively, aminooxy, hydrazine or hydrazide labels, such as aminooxyTMT (Zhong et al. 2015) and Girard’s reagent P (Walker et al. 2011), enable rapid N-glycan labeling as they react with the reducing end aldehyde, but do not require a reduction step for stability. A common feature of the RapiFluorMS, InstantPC, aminooxyTMT and procainamide label is that they carry a tertiary amine, facilitating protonation for positive mode MS. This feature results in an advantage in ionization efficiency (and thus sensitivity), as compared to the amide present on the 2-Ab label, enabling the analysis of released glycans from less than 5 µg of IgG (Keser et al. 2018). In addition to the presence of a basic group, higher hydrophobicity of the labels improves ionization efficiency in ESI by a more efficient desolvation of the labeled products (Walker et al. 2011).

The separation of released glycans prior to MS analysis aids their in-depth structural characterization. HILIC-LC is a robust and broadly applied separation technique for MS-based glycan analysis, especially in the biopharmaceutical setting. An increasing number of HILIC-LC-compatible reducing end labels is reported that enable sensitive glycan analysis by positive mode MS. Prominent examples are RapiFluorMS (Lauber et al. 2015), InstantPC (Kimzey et al. 2015) and procainamide (Kozak et al. 2015). The use of RapiFluorMS labeling in combination with HILIC-LC-MS/MS recently proved to be a powerful method for the structural characterization of mAb glycosylation, identifying Gal-α1,3-Gal isomers on a National Institute of Standards and Technology (NIST) reference material mAb (Hilliard et al. 2017). As compared to HILIC-LC, isomer separation by PGC-LC is even more powerful, as it separates the isomers of neutral and acidic glycoforms simultaneously. For example, N-glycans carrying a bisecting GlcNAc, as present on IgG and IgA, elute earlier as compared to non-bisected glycans with an extra antenna. Additionally, fucose and sialic acid isomers can be separated (Stadlmann et al. 2008; Kolarich et al. 2015; Abrahams et al. 2018). PGC-LC is commonly used in combination with negative mode tandem MS for the structural elucidation of isomeric glycans (Everest-Dass et al. 2013; Kolarich et al. 2015). A recent analysis of released glycans of IgG by TiO2-PGC-LC-MS/MS reported the presence of a high number of isomeric N-glycan structures, including sulfated glycoforms (Wang et al. 2017, 2018). This method relied on the selective enrichment of acidic glycoforms on the TiO2-trap column and reported associations between a selected group of the trace glycans and rheumatoid arthritis. Notably, when in-depth analyses on minor species are performed on the level of released glycans, it is advisable to perform orthogonal proteomic as well as glycoproteomic analyses at the glycopeptide level, in order to rule out that glycomic results on minor species are confounded by minor amounts of contaminating glycoproteins (Lauc et al. 2018).

Alternative to chromatographic separations, CE efficiently separates carbohydrates based on their charge and size. Coupling CE to MS via ESI provides low-flow nano-ESI conditions and results in high sensitivity for in-depth glycomics (Lageveen-Kammeijer et al. 2019). Similarly to HILIC-LC-ESI-MS approaches, reducing end glycan labeling improves the ionization efficiency for CE-ESI-MS. Additionally, the introduction of a charged label to the neutral species allows their electrophoretic migration in CE. For example, the use of positive labels (e.g. aminooxyTMT or Girard’s reagent P), in combination with either a low pH background electrolyte or the neutralization of sialylated species, facilitates normal polarity CE separation in combination with positive mode MS analysis (Zhong et al. 2015; Khatri et al. 2017). While currently not broadly used for the characterization of antibody glycosylation, CE-LC-MS does provide a promising method for the sensitive separation of isomeric glycan structures derived from immunoglobulins. This includes the possibility to multiplex the analysis, when labeling with a tandem mass tag (TMT) is performed (Zhong et al. 2015).

One of the most recent developments of N-glycan isomer characterization focuses on the separation of reduced N-glycans in the gas phase by IMS-MS. Separation by IMS-MS is based on the charge and shape of the ions, which can be converted to their collision cross section (CCS; Harvey et al. 2016, Glaskin et al. 2017). The recent publication of a library containing N-glycan CCSs from standard proteins, including IgG, helps to assign glycan isomers with IMS-MS (Struwe et al. 2016). However, the separation power of IMS-MS for N-glycans is still limited and further complicated by the existence of glycan conformers (Struwe et al. 2015). Likely, technical and computational modeling developments will enhance the power of this technique for N-glycan characterization in the future. In the meantime, the additional resolution of separation techniques, such as CE, can help to differentiate isomers from conformers (Jooss et al. 2018).

**Glycopeptides**

In contrast to released glycan analysis, the analysis of antibody glycosylation at the glycopeptide level usually achieves significant protein specificity. Additionally, when only one site is present per peptide, this approach allows site-specific analysis. General glycopeptide-based workflows include the isolation of the antibodies, their cleavage with a proteolytic enzyme and enrichment or online separation of the glycopeptides followed by their analysis by MS (AMS). Online separation is often achieved by hyphenating LC or CE to MS via ESI (Heemskerk et al. 2013; Stavenhagen, Plomp, Wuhrer, et al. 2015; Falck et al. 2017; Plomp et al. 2018; Chandler et al. 2019). Alternative methods include off-line enrichment of glycopeptides in combination with their matrix-assisted laser desorption/ionization (MALDI)-MS analysis (de Haan et al. 2015; Bondt et al. 2016; Wu et al. 2016; Bondt et al. 2017).

For the identification of glycosylation sites and the determination of site occupancy, glycopeptides are often treated by the enzyme PNGase F, which releases the N-glycans and converts the asparagines, to which the glycans were linked, into aspartic acids (Stavenhagen,
treated IgG3 samples (Plomp et al. 2015). More recently, also the \(\alpha\)2,3-linked sialylated species were characterized from IgA derived from plasma, colostrum and saliva, including the glycosylation profiles on the J-chain and secretory component associated with secretory IgA (Deshpande et al. 2010; Plomp et al. 2018; Chandler et al. 2019). These studies revealed body fluid-specific glycosylation profiles, which indicate that careful biofluid selection is an important factor in biomarker discovery (Deshpande et al. 2010; Plomp et al. 2018). IgM purified from human plasma was recently characterized in a site-specific manner, assessing not only the different glycoforms at the five distinct N-glycosylation sites, but also the occupancy of these sites. While four of the sites were almost completely occupied, the C-terminal N439 showed an occupancy of only 30–40% (Chandler et al. 2019).

LC separation techniques complementary to RP-LC include HILIC- and PGC-LC. HILIC-LC is especially suitable for the separation of glycopeptides with short peptide sequences, which may not be retained by RP-LC, and it allows the efficient separation of glycosylated and non-glycosylated peptides in complex mixtures (Zauner et al. 2010). Additionally, for sialylated glycopeptides, HILIC enables the differentiation between \(\alpha\)2,3- and \(\alpha\)2,6-linked sialic acids, showing \(\alpha\)2,6-linked sialic acids to have a higher retention on a zwitterionic type-HILIC (ZIC-HILIC) stationary phase as compared to \(\alpha\)2,3-linked sialylated species (Takegawa et al. 2006).

Similar to HILIC, PGC-LC is able to separate glycopeptides with short peptide moieties, with the added advantage that samples do not have to be loaded in high concentrations of organic solvent. However, it should be taken into account that both highly sialylated glycopeptides and glycopeptides with longer peptide sequences might be irreversibly retained on the PGC stationary phase (Stavenhagen, Kolarich, Wührer, et al. 2015). The same behavior is observed for hydrophobic matrix components, e.g., lipids or hydrophobic peptides, to a much higher degree than in RP-LC. Consequently, PGC requires (more) elaborate sample pre-purification. As the three mentioned chromatographic approaches have complementary properties, combinations are used for the characterization of immunoglobulin glycopeptides (Liu et al. 2014; Stavenhagen, Plomp, Wührer, et al. 2015). For example, sequential C18-PGC-LC-MS was used to study the C4\(\alpha\)3 domain N-glycosylation site of IgG3, after treatment of IgG3 with trypsin and AspN. This revealed higher levels of non-fucosylated glycans at this site as compared to the conserved glycosylation site in the C4\(\alpha\)2 domain (Stavenhagen, Plomp, Wührer et al. 2015).

The separation mechanism of CE is complementary to RP-LC as IgG Fc glycopeptides are mainly separated based on their glycan moiety, resulting in the co-migration of identical glycoforms attached to different subclasses (Heemskerk et al. 2013). However, due to the size- and charge-based separation, CE may also be complementary to HILIC-LC when the differences between peptide portions are larger. Furthermore, as reported for released glycans, CE-ESI-MS is capable of separating N-glycan sialic acid isomers on the IgG glycopeptide level, showing a faster migration for \(\alpha\)2,6-linked sialylated species as compared to \(\alpha\)2,3-linked sialylated species (Kammeier et al. 2017). Notably, CE-ESI-MS is an extremely sensitive technique, able to detect glycopeptides derived from IgG at low picogram amounts loaded into the capillary (Kammeier et al. 2016). Efficient, high sensitivity sample preparation methods are now needed in order to make ultrahigh-sensitivity CE-MS available for the characterization of glycopeptides derived from, for example, the very low abundance plasma IgE.

For IgG Fc glycosylation, usually trypsin is used to digest the protein. Human IgG consists of four subclasses (IgG1–4), which have their specific biological activities as well as slightly different glycosylation. Obtaining IgG glycopeptides with peptide moieties fully specific for the IgG subclass is not trivial. While the tryptic digestion of IgG1, 2 and 4 always results in distinct peptide portions (independent of the allotypes; Table II), and can thus be separated with MS, the numerous known IgG3 allotypes vary in their peptide sequences surrounding the C4\(\alpha\)2 glycosylation site. For 2 of the 19 IgG3 allotypes full tryptic cleavage is expected to result in a peptide portion identical to the peptide portion of IgG2 (EEQYNSTFR; Table II), while most of the others are isomers of the IgG4 peptide (EEQYNSTFR; Table II) and only two of them result in a longer, unique peptide (TKPWEEQYNSTFR; Table II; Vidarsson et al. 2014). A similar situation applies for certain allotypes of the two subclasses of IgA, for which the peptide moieties surrounding the conserved glycosylation sites in the C4\(\alpha\)2 and C4\(\alpha\)3 domains are identical after tryptic digestion (Plomp et al. 2018; Table II). These challenges might be partially overcome by the use of proteases with a different specificity as compared to trypsin, as they might result in larger and more specific protein fragments. On the basis of available protein sequences and known enzyme specificities, GluC, which preferably cuts proteins C-terminal of glutamic acid and aspartic acid residues, may be a particularly promising candidate for complementing trypsin in IgG subclass- and allotype-specific glycopeptide analysis.

The in-depth site-specific glycosylation analysis of IgG3, IgA1 and IgD hinge region O-glycosylation remains a challenging task (Wada et al. 2010). This is due to the resistance of these heavily O-glycosylated regions to proteolytic digestion and the low number of cleavage sites available in these regions, often resulting in glycopeptides with multiple glycosylation sites. Recent developments in the area of O-glycan proteases might help to overcome these limitations, by enabling the cleavage at the N-terminus of O-glycosylated serines or threonines. The potential of such an O-glycan protease was recently shown for other O-glycosylated proteins, either used in stand-alone mode or in combination with another protease, like trypsin (Yang, Ao et al. 2018, Yang, Onigman, et al. 2018).
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As LacyTools (Jansen et al. 2016). Alternatively, targeted tandem MS data extraction can be performed with dedicated software tools, such as targeted approaches. Targeted tandem MS methods are well suited for complex samples and were recently described for the relative quantification of IgG, IgA and IgM glycopeptides from plasma, with inclusion of additional, more specific transitions such as glycopeptide Y-ions is advisable in order to secure the specificity of these targeted transitions are then monitored by selecting a product ion either in the second mass analyzer (SRM) or during data processing (PIA).

Table II. Peptide moieties, and their masses, of the tryptic glycopeptides of human IgG and IgA

| Protein      | Glycosylation site | UniProt number | Tryptic peptide sequence (glycosylation site) | Peptide mass [M] (Da) |
|--------------|--------------------|----------------|---------------------------------------------|----------------------|
| Human IgG1   | N297               | N180           | EEQYNSTYR                                   | 1188.5047            |
| Human IgG2   | N297               | N176           | EEQFNSTFR                                   | 1156.5149            |
| Human IgG3   | 7/7/7/7/7/7/7/7/7/7 | T122/T137/T152 | SCDTPPPCPR                                  | 1071.4478            |
| Human IgG3   | N297               | N227           | EEQFNSTFR$^a$                               | 1156.5149$^a$        |
| Human IgG4   | N297               | N177           | EEQYNSTFR$^a$                               | 1172.5098$^a$        |
| Human IgA1   | T225/T228/S230/S232/T233/T236/S1117 | S117/T114/T117 | HYTNPSQDVTVPVPSTP                           | 4388.9372            |
| Human IgA1   | N263               | N144           | LSLLRPAEDLLLGSEANLTCCTLTGLR                 | 2962.5909            |
| Human IgA1   | N459               | N340$^b$       | LAGKPTHVINSVVMAEVGTC(Y)$^b$                 | 2183.0714            |
| Human IgA2   | N166               | N47            | VFLSPLSTQDNVVVACLVQ                         | 4355.8826            |
| Human IgA2   | N211               | N92$^c$        | HYTNSSQDVTVPCR$^c$                          | 5333.2526           |
| Human IgA1   | N263               | N131           | LSLHRPAEDLLLGSEANLTCCTLTGLR                 | 2962.5909            |
| Human IgA2   | N337               | N205           | TPLTANITK                                   | 957.5495             |
| Human IgA2   | N459               | N327$^b$       | LAGKPTHVINSVVMAEVGTC(Y)$^b$                 | 2183.0714$^b$        |
|              |                    |                | MAGKPTHVINSVVMAEADGTC(Y)$^b$                | (2346.1348)$^b$      |

Both MS- and tandem MS-based glycopeptide methods are used for the (relative) quantitative profiling of immunoglobulin glycosylation. When no fragmentation is performed, glycopeptides are identified based on accurate mass and isotopic pattern, and targeted data extraction can be performed with dedicated software tools, such as LacyTools (Jansen et al. 2016). Alternatively, targeted tandem MS methods, like selected reaction monitoring (SRM) or product-ion analysis (PIA), also sometimes called parallel reaction monitoring, can be used. In both modes, strongly linked to unit mass resolution (quadrupole or ion trap) or high resolution (time-of-flight, orbitrap) mass analyzers, respectively, only the ion of interest (precursor) is transferred to the collision cell. A specific transition is then monitored by selecting a product ion either in the second mass analyzer (SRM) or during data processing (PIA). Usually, an intense oxonium ion is selected during SRM, while the inclusion of additional, more specific transitions such as glycopeptide Y-ions is advisable in order to secure the specificity of these targeted approaches. Targeted tandem MS methods are well suited for complex samples and were recently described for the relative quantification of IgG, IgA and IgM glycopeptides from plasma, without the requirement of an immunoglobulin enrichment step (Hong et al. 2015; Yuan et al. 2015). The application of such a method on a clinical cohort of epithelial ovarian cancer patients showed the differential expression of glycopeptides from all of these antibodies (Ruhak et al. 2016). For both targeted and untargeted MS methods, the relative quantification of glycoforms is dependent on some form of normalization. Most often, total area normalization is used, which has as a downside that the abundancies of individual glycoforms observed are not independent, i.e. when the absolute abundance of one glycoform increases, other glycoforms will decrease in relative abundance although their absolute abundance is unchanged. Additionally, while differences in ionization and detection efficiency of glycopeptides with the same peptide backbone can be minimized in MS, intrinsic differences in their fragmentation result in large biases in relative tandem MS analysis and make total area normalization ill-suited for tandem MS approaches. The independent quantification of glycoforms can be obtained by using heavy isotope labeled internal standards. Such standards have the additional potential to improve method robustness. While an ideal internal standard would cover the complete repertoire of different glycoforms per immunoglobulin glycosylation site, obtaining these standards is still challenging. Recent attempts focused on the use of heavy-labeled IgG glycopeptides containing only one GlcNAc for the quantification of IgG subclasses and glycoforms (Roy et al. 2018). Alternatively, glycopeptide functional groups were employed for the introduction of an external heavy (for the internal standard) or light (for the studied samples) label to the glycoforms (Roy et al. 2018). Alternatively, glycopeptide functional groups were employed for the introduction of an external heavy (for the internal standard) or light (for the studied samples) label to the analytes, using, for example, heavy-labeled variants of the amine reactive benzoic acid N-hydroxysuccinimide, succinic anhydride or TMT (Ye et al. 2013; Kurogochi and Amano 2014; Pabst et al. 2016). The latter was never described for its use with immunoglobulin glycopeptides, but provides the opportunity to multiplex up to 10 samples by labeling them with different isobaric tags which allows their quantification after MS fragmentation (Ye et al. 2013). Another strategy was recently used for the comparison between monoclonal...
antibody glycosylation of biosimilars and their innovator product by modifying the C-terminus of glycopeptides with $^{18}$O during the tryptic digestion of the protein in H$_2^{18}$O (Srikanth et al. 2017). Finally, the development of intact heavy-labeled antibodies might be a suitable source of heavy-labeled glycopeptides in the future, especially when their glycosylation resembles human antibody glycosylation. Intact protein standards have the advantage over glycopeptide standards that they can be introduced in the sample preparation protocol at an early stage, enabling correction for additional sample processing steps.

**Intact glycoproteins and large protein fragments**

The analysis of intact glycoproteins by MS is a powerful method for the characterization of proteoforms, including the combination of different glycosylation sites and other post-translational modifications (PTMs). Although this is a substantial advantage over the methods described above, the large glycan heterogeneity in combination with the numerous charge states in which the intact glycoproteins occur in ESI-MS make in-depth analysis challenging. Additionally, efficient tandem MS of intact antibodies is still in its infancy and the resolving power of commonly used separation methods is less developed for glycoproteins as compared to glycopeptides. This means that, similar to released glycan analysis, site-specificity is often lost. Luckily, new developments addressing these challenges have recently accelerated the intact and top-down analysis of monoclonal IgGs (Periat et al. 2016; Toby et al. 2016; Bobaly et al. 2018; Goyon, Francois, et al. 2018; van der Burgt et al. 2019). Additionally, so-called middle-up and middle-down approaches, in which the Fab portion is cleaved from the Fc portion prior to MS analysis, provide another solution for the more in-depth characterization of IgG and its PTMs (Resemann et al. 2016).

The major technical developments in the field of intact antibody characterization involved new and improved high resolution MS methods (Toby et al. 2016). MS approaches generally used include Orbitrap-MS (Rosati et al. 2012), Fourier transform ion cyclotron resonance (FTICR)-MS (Nicolardi et al. 2014) and high resolution time-of-flight (TOF)-MS (Haselberg et al. 2018). Tandem MS of intact glycoproteins is complicated by the fact that fragmentation strategies which are commonly used for glycopeptides, like CID, ETD and ECD (or combinations thereof), often result in inefficient fragmentation. However, using Orbitrap-MS in combination with ETD and ETnCD (a combination of electron transfer dissociation and higher energy collisional dissociation) after ESI, fragmentation was achieved of therapeutic IgG1 and IgG2 molecules, showing a sequence coverage of approximately 30% (Fornelli et al. 2017). Alternatively, using MALDI-in-source decay-FTICR-MS for a NIST monoclonal antibody reference material, 31% and 65% sequence coverage was achieved for the heavy and the light chain, respectively (van der Burgt et al. 2019).

Intact antibody samples as well as antibody-based biopharmaceuticals can be directly analyzed by MALDI-MS or via direct infusion with ESI-MS (Yang et al. 2017; Wohlschläger et al. 2018; van der Burgt et al. 2019). However, proteoforms are often (partly) separated prior to ESI-MS by LC or CE to reduce the complexity of the MS data. Various LC modes hyphenated to MS are reported for the intact or top-down characterization of mAbs, including RP-LC and HILIC (Periat et al. 2016; Bobaly et al. 2018). While RP-LC provides efficient separation of mAbs based on their hydrophobicity and is often used to assess biopharmaceutical protein degradation or misfolding (Rathore 2009), HILIC is well suited for the analysis of glycoproteins as it allows glycoform separation and provides a high sensitivity with MS (Periat et al. 2016). Alternative LC methods for glycoprotein separation include size exclusion chromatography (SEC), ion-exchange chromatography and hydrophobic interaction chromatography (Fekete et al. 2017). Additionally, these can be used as first dimension in 2D-LC-MS (Fekete et al. 2017). For example, SEC is very powerful for the characterization of protein aggregation and may precede RP-LC for MS analysis (Goyon, Sciascera, et al. 2018). An exciting recent development is the coupling of affinity chromatography to MS, providing information on the interaction of specific proteoforms of IgG with, for example, the Fc neonatal receptor (Gahoual et al. 2017). Besides LC, CE is also highly suitable for intact mAb separation, providing information on antibody charge variants including deamidated products (Goyon, Francois, et al. 2018; Haselberg et al. 2018).

Sample preparation for the intact analysis of IgGs often involves the purification and desalting of the antibody prior to injection into the system. Alternatively, to reduce complexity, specific enzymes may be used that cleave in between the Fab and Fc portion, or disulfide bridges of the antibody may simply be reduced, yielding the two light and the two heavy chains separately. The MS profiling of the resulting fragments is referred to as middle-up, while the subsequent MS fragmentation of the protein fragments, to obtain sequence information, is called middle-down (Resemann et al. 2016). Examples of enzymes used in these approaches are IdES, which is expected to result in two Fc/2 fragments and one intact F(ab’)/2 fragment by cleaving IgG under the hinge region, and GingisKHAN which results in one intact Fc and two intact Fab fragments by cleaving IgG above the hinge region (van der Burgt et al. 2019). Both MALDI-MS and direct infusion ESI-MS perform quite well for the profiling of IgG Fc glycoforms, providing similar results to released glycan and glycopeptide methods, albeit with less analytical depth in terms of the number of glycoforms covered (Reusch, Haberger, Falck, et al. 2015; van der Burgt et al. 2019). Besides obtaining higher resolution both in separations as well as mass analysis (Stoll et al. 2018), the cleavage of Fc and Fab is especially useful for Fab glycosylated antibodies. For example, for the marketed therapeutic mAb Cetuximab a significant reduction in data complexity was reported after treatment with IdeS (Dai and Zhang 2018). Additionally, it enables the characterization of polyclonal Fc glycosylation by removing the hyper variable Fab portion (Leblanc et al. 2014). Finally, the middle-up characterization of IdES digested antibodies by HILIC-ESI-MS proved to be suitable for monitoring drug-antibody coupling for antibody-drug conjugates (D’Arriet al. 2018). Approaches similar to the ones described above for IgG may be suitable for the middle-up or middle-down characterization of IgA1 and IgD glycosylation using the O-glycan proteases reported to cleave N-terminal of O-glycosylation sites (Yang, Ao, et al. 2018). Such an enzyme would be able to separate Fc from Fab of these antibodies by cleaving in the hinge region.

**Other approaches**

Traditionally, glycans, or better glycan motifs, have been analyzed with lectins or anti-carbohydrate antibodies (Table I). Due to the low specificity, especially of lectins, this has often made conclusions difficult or has even led to misinterpretations (Hendrickson and Zherdev 2018). Nonetheless, the simplicity and low implementation hurdle make the approach attractive, especially for routine applications, if drawbacks can be managed. For example, lectin microarrays can achieve increased specificity by relying on multiple binding hits to lectins with overlapping specificities (Cook et al. 2015). Additionally,
they can be used in fingerprinting approaches focusing on emerging differences, rendering specificity less important, as demonstrated for the differentiation of therapeutic mAb critical quality attributes (Zhang et al. 2016). Increasing the specificity of individual affinity probes by chemical biology is another approach which employs, for example, engineered lectins and catalytically silent glycosidases (Arnaud et al. 2013; Lectenz® Bio 2019).

Glycoengineering has opened up many new possibilities to study structure–function relationships of antibody glycosylation. In recent years, several groups have engaged in extensive glycoform-resolved functional studies using biochemical instead of molecular detection. Surface plasmon resonance (SPR) and affinity chromatography with UV detection have been used to unravel the complex influence of IgG Fc glycans on receptor binding, including Fc gamma receptor (FcγR), fetal/neonatal Fc receptor and complement factors (Dashiwets et al. 2015; Thomann et al. 2015; Subedi and Barb 2016; Dekkers et al. 2017; Wada et al. 2019; Table I). This was often complemented with biological assays for antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity, confirming the glycan impact on these important immunological responses.

A strong three-dimensional structural basis for these studies has been provided through the crystallographic analysis of IgG alone and in complex with FcγR. This remarkably showed glycan–glycan and glycan–protein interactions within IgG and between IgG and the FcγR (Krapp et al. 2003; Ferrara et al. 2011). In recent years, this basis has been refined by protein nuclear magnetic resonance spectroscopy (NMR) studies (Table I). With a consequential focus on solution phase structures and more flexible protein regions, the impact of the IgG Fc glycan on the organization of the N139Y-containing Cε loop has been highlighted as a critical factor for FcγR binding (Subedi and Barb 2015). Additionally, the structural basis for the outstanding role of IgG Fc core fucosylation has been refined (Falconer et al. 2018). Based on the detailed findings of the IgG glycan functional impact and indications of the FcγR glycan importance in its interaction with IgG (Hayes et al. 2014), natural human FcγR glycosylation and the functional impact of FcγR glycoforms are becoming a focus of attention (Hayes et al. 2017; Patel et al. 2018; Yagi et al. 2018; Washburn et al. 2019).

**Perspectives**

Most research on antibody glycosylation is performed for endogenous IgG derived from plasma or recombinant IgG from culture broths in the biopharma industry. IgG is the most abundant antibody isotype in the human circulation and a lot is known about its role in the humoral immune system. Furthermore, there are many IgG-based biopharmaceuticals and the number continues to grow. However, this does not mean that the glycosylation analysis of other immunoglobulin isotypes, or of antibodies derived from other body fluids, is less important. That still little is known on the behavior and effect of the glycosylation of antibodies other than IgG in health or disease is likely a consequence of their lower abundance in the circulation and the higher complexity of their glycosylation. Furthermore, sometimes it may be more relevant to study antibodies in biofluids other than plasma, such as saliva, synovial fluid or cerebrospinal fluid, or even in tissue. However, this is hampered by low concentrations or poor accessibility of these samples.

Recent developments in MS-based technologies, like the high sensitivity nano-LC-ESI-MS and CE-ESI-MS analysis of glycopeptides, provide the platforms to bring the field of global antibody glyco-proteomics further. Steps should be taken for the miniaturization of the sample preparation, most importantly for low abundance antibodies like IgD and IgE, of which the latter specifically is gaining more and more interest in the field of allergy research (Shade et al. 2019). Furthermore, miniaturization of the sample preparation is of value when antigen-specific sub-populations of antibodies are studied (Kapur et al. 2014) as well as for antibodies derived from small-scale clonal in vitro cultures. The latter may provide valuable insights into the regulation of immunoglobulin microheterogeneity.

Similar to IgG, IgA glycosylation can currently be routinely profiled at the glycopeptide level in biomedical settings (Plomp et al. 2018; Chandler et al. 2019). Applications of this method may be particularly relevant for diseases with mucosal involvement, such as inflammatory bowel diseases and colon cancer, where the glycosylation of secretory IgA can be compared to its blood counterpart.

While glycopeptide analysis is key in the site-specific characterization of immunoglobulin glycosylation, challenges remain with respect to the full structural characterization of all glycoforms as well as comprehensive proteoform analysis. The prior can be addressed by combining a glycopeptidomic approach with a glycomic approach, releasing the glycans from purified immunoglobulins and subjecting them to powerful isomer separation techniques like PGC-LC or CE prior to tandem MS. The current developments in IMS may very well lead to its integration in such characterization workflows, both on the glycan and glycopeptide level. Especially, the smart integration of IMS with liquid phase separations, mass spectrometry and fragmentation techniques shows great promises of orthogonalism for structural analysis. Comprehensive proteoform analysis can be addressed by MS-based intact and middle-up approaches. On the level of glycosylation, we expect a method that allows the analysis of an intact Fc portion, for example via IgG digestion by GensisKHAN, to provide valuable information on the combinations of glycoforms present on the heavy chain Fc dimer. Additionally, information will be obtained regarding other PTMs of the antibody, such as oxidation, deamidation, glycation and proteolytic truncation that may influence immunoglobulin effector functions and half-life. However, classical peptide mapping, which is modified to accurately include PTMs, will be essential to warrant the site-specific analysis of co-occurring PTMs (Choi et al. 2017). While this may also be efficiently addressed by top-down or middle-down approaches in the far future, these are unlikely to provide a sole solution for all relevant PTMs.

To advance the in-depth and high-throughput screening of high numbers of samples in biomedical research, progress should be made in the robustness and automation of current methods. Additionally, as antibody glycosylation is expected to play a role in future clinical diagnostics, efforts should be made to simplify current workflows and adjust them to clinical diagnostic platforms that are currently in use. Here, one can think about simplification of sampling methods, for example by using dried blood spots (Gudelj et al. 2015; Choi et al. 2017). While this may also be efficiently addressed by top-down or middle-down approaches in the far future, these are unlikely to provide a sole solution for all relevant PTMs.
may be gained by the integrated analysis of different immunoglobulin classes. This was shown to be possible via the direct digestion of the complete plasma proteome, in combination with a targeted MS approach for IgG, IgA and IgM glycopeptides (Hong et al. 2015). Alternatively, we expect a more in-depth characterization of immunoglobulin glycosylation microheterogeneity when various antibodies are simultaneously enriched from the same sample prior to glycopeptide generation.

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**Conflict of interest statement.**

The authors declare no conflict of interest.

**Abbreviations**

2-AB, 2-aminobenzamide; AC-UV, affinity chromatography with UV detection; AEX, anion exchange; APTS, aminopryene trisulfonate; CCS, collision cross section; CGE-LIF, capillary gel electrophoresis with laser-induced FLU; CID, collision-induced dissociation; CZE, capillary zone electrophoresis; ECD, electron-capture dissociation; ESI, electrospray ionization; ETD, electron-transfer dissociation; FLU, fluorescence; FTICR, Fourier transform ion cyclotron resonance; HIIC, hydrophilic interaction liquid chromatography; IMS, ion mobility spectrometry; mAbs, monoclonal antibodies; MS, mass spectrometric; PGC, porous graphitized carbon; PIA, production analysis; RP, reversed phase; SEC, size exclusion chromatography; SPR, Surface plasmn resonance; SRM, selected reaction monitoring; TMT, tandem mass tag.

**References**

Abrahams JL, Campbell MP, Packer NH. 2018. Building a PGC-LC-MS N-glycan retention library and elution mapping resource. Glycocon J. 35:15–29.

Ackerman ME, Crispin M, Yu X, Baraah K, Boesch AW, Harvey DJ, Dugast AS, Heizen EL, Ercan A, Choi et al. 2013. Natural variation in Fe glycosylation of HIV-specific antibodies impacts antiviral activity. J Clin Invest. 123:2183–2192.

Arnold J, Audfray A, Imberty A. 2013. Binding sugars: From natural lectins to synthetic receptors and engineered neolactins. Chem Soc Rev. 42:4798–4813.

Arnold JN, Wormald MR, Sim RB, Rudd PM, Dwek RA. 2007. The impact of glycosylation on the biological function and structure of human immunoglobulins. Annu Rev Immunol. 25:21–50.

Bobaly B, D’Atri V, Lauher M, Beck A, Guillarome D, Fekete S. 2018. Characterizing various monoclonal antibodies with milder reversed phase chromatography conditions. J Chromatogr B Analyt Technol Biomed Life Sci. 1096:1–10.

Bondt A, Nicolardi S, Jansen BC, Kuiper TM, Hazes JMW, van der Burg YM, Wurher M, Dolhain R. 2017. IgA N- and O-glycosylation profiling reveals no association with the pregnancy-related improvement in rheumatoid arthritis. Arthritis Res Ther. 19:160.

Bondt A, Nicolardi S, Jansen BC, Stavenhagen K, Blank D, Kammeijer GS, Kozak RP, Fernandes DI, Hensbergen PJ, Hazes JMW et al. 2016. Longitudinal monitoring of immunoglobulin A glycosylation during pregnancy by simultaneous MALDI-FTICR-MS analysis of N- and O-glycopeptides. Sci Rep. 6:27955.

Bondu A, Rombouts Y, Selman MH, Hensbergen PJ, Reiding KR, Hazes JM, Dolhain RJ, Wurher M. 2014. Immunoglobulin G (IgG) Fab glycosylation analysis using a new mass spectrometric high-throughput profiling method reveals pregnancy-associated changes. Mol Cell Proteomics. 13:3029–3039.

Borza B, Szegi M, Szekrenyes A, Hajba L, Guttmann A. 2018. Glycosimilarity assessment of biotherapeutics 1: Quantitative analysis of the N-glycosylation of the innovator and a biosimilar version of etanercept. J Pharm Biomed Anal. 153:182–183.

Chandler KB, Mehta N, Leon DR, Suscovich T, Alter G, Costello CE. 2019. Multi-isotype glycoproteomic characterization of serum antibody heavy chains reveals isotype- and subclass-specific N-glycosylation profiles. Mol Cell Proteomics. 18:686–703.

Choi NY, Hwang H, Ji ES, Park GW, Lee JY, Lee HK, Kim JY, Yoo JS. 2017. Direct analysis of site-specific N-glycopeptides of serological proteins in dried blood spot samples. Anal Bioanal Chem. 409:4971–4981.

Colhoun HO, Treacy EP, MacMahon M, Rudd PM, Fitzgibbon M, O’Flaherty R, Stepieen KM. 2018. Validation of an automated ultraperformance liquid chromatography IgG N-glycan analytical method applicable to classical galactosaeemia. Ann Clin Biochem. 55:593–603.

Cook MC, Kaldas SJ, Muradia G, Rosu-Myles M, Kunkel JP. 2015. Comparison of orthogonal chromatographic and lectin-affinity microarray methods for glycan profiling of a therapeutic monoclonal antibody. J Chromatogr B Analyt Technol Biomed Life Sci. 997:162–178.

D’Atri V, Fekete S, Stoll D, Lauber M, Beck A, Guillarome D. 2018. Characterization of an antibody-drug conjugate by hydrophilic interaction chromatography coupled to mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci. 1080:37–41.

Dai J, Zhang Y. 2018. A middle-up approach with online capillary isoelectric focusing/mass spectrometry for in-depth characterization of Cetuximab charge heterogeneity. Anal Chem. 90:14527–14534.

Dashivets T, Thomann M, Rueger P, Knaupp A, Buchner J, Schlothauer T. 2015. Multi-angle effector function analysis of human monoclonal IgG glycovariants. PLoS One. 10:e0143520.

de Haan N, Reiding KR, Haberger M, Reusch D, Falck D, Wurher M. 2015. Linkage-specific sialic acid derivatization for MALDI-TOF-MS profiling of IgG glycopeptides. Anal Chem. 87:8284–8291.

de Haan N, Reiding KR, Kristic J, Hippigrave Ederveen AL, Lauc G, Wurher M. 2017. The N-glycosylation of mouse immunoglobulin G (IgG)-fragment crystallizable differs between IgG subclasses and strains. Front Immunol. 8:608.

Dekkers G, Pomp R, Koelman CA, Visser R, von Horsten HH, Sandig V, Rispens T, Wuhrer M, Vidarsson G. 2016. Multi-level glyco-engineering techniques to generate IgG with defined Fc-glycans. Sci Rep. 6:36964.

Dekkers G, Trefers L, Pomp R, Bentlage AEH, de Boer M, Koelman CAM, Lissenberg-Thunnissen SN, Visser R, Brouwer M, Mok JY et al. 2017. Decoding the human immunoglobulin G-glycan repertoire reveals a spectrum of Fc-receptor- and complement-mediated-effector activities. Front Immunol. 8:877.

Deshpande N, Jensen PH, Packer NH, Kolarich D. 2010. GlycoSpectrumScan: Fishing glycopeptides from MS spectra of protease digests of human colostrum slgA. J Proteome Res. 9:1063–1075.

Dicker M, Tschofen M, Maresch D, Konig J, Juarez P, Orzaez D, Altmann F, Steinkellner H, Strasser R. 2016. Transient glyco-engineering to produce recombinant IgA1 with defined N- and O-glycans in plants. Front Plant Sci. 7:18.

everest-Dass AV, Abrahams JL, Kolarich D, Packer NH, Campbell MP. 2013. Structural feature ions for distinguishing N- and O-linked glycan isomers by LC-ESI-IT MS/MS. J Am Soc Mass Spectrom. 24:895–906.

Falck D, Jansen BC, de Haan N, Wurher M. 2017. High-throughput analysis of IgG Fc glycopeptides by LC-MS. Methods Mol Biol. 1503:31–47.

Falconer DJ, Subedi GP, Marcella AM, Barb AW. 2018. Antibody fucosylation lowers the FcγRgammaIIb/CD16a affinity by limiting the conformations sampled by the N162-fucosyl. ACS Chem Biol. 13:2179–2189.

Fekete S, Veuthey JL, Guillarome D. 2017. Achievable separation performance and analysis time in current liquid chromatographic practice for monoclonal antibody separations. J Pharm Biomed Anal. 141:59–69.
Ferrara C, Grau S, Jager C, Sondermann P, Brunker P, Waldhauer I, Henning M, Ruf A, Rufer AC, Stehle M et al. 2011. Unique carbohydrate-protein interaction are required for high affinity binding between Fc gamma RIII and antibodies lacking core fucose. *Proc Natl Acad Sci USA.* 108:12669–12674.

Fornelli L, Ayoub D, Aizikov K, Liu X, Damoc E, Pezner PA, Makarov A, Beck A, Tsybin YO. 2017. Top-down analysis of immunoglobulin G isoforms and 2 with electron transfer dissociation on a high-field Orbitrap mass spectrometer. *J Proteomics.* 159:67–76.

Gahoual R, Heidenreich AK, Somsen GW, Bulau P, Reusch D, Wuhrer M, Giddens JP, Lomino JV, DiLillo DJ, Ravetch JV, Wang LX. 2018. Site-selective monitoring of immunoglobulin glycosylation. *237*

Goyon A, Sciascera L, Clarke A, Guillarme D, Pell R. 2018. Extending the limits of size exclusion chromatography: Simultaneous separation of free and bound glycoproteins. *Cell Immunol.* 354:204–212.

Gennaro LA, Delaney J, Vorov P, Harvey DJ, Domon B. 2002. Capillary electrophoresis/electrospray ion trap mass spectrometry for the analysis of negatively charged derivatized and underivatized glycans. *Rapid Commun Mass Spectrom.* 16:192–200.

Giddens JP, Lomino JV, DiLillo DJ, Ravetch JW, Wang LX. 2018. Site-selective chemoenzymatic glycoengineering of Fab and Fc glycans of a therapeutic antibody. *Proc Natl Acad Sci U S A.* 115:12023–12027.

Glaskin RS, Khatri K, Wang Q, Zaia J, Costello CE. 2017. Construction of a database of collision cross section values for glycans, peptides, and glycopeptides determined by IM-MS. *Anal Chem.* 89:4432–4460.

Goh JB, Ng SK. 2018. Impact of host cell line choice on glycan profile. *Citr Rev Biotechnol.* 38:831–867.

Goyon A, Francois YN, Colas O, Beck A, Veuthey JL, Guillarme D. 2018. High-resolution separation of monoclonal antibodies mixtures and their charge variants by an alternative and generic CE method. *Electrophoresis.* 39:2083–2090.

Goyon A, Szascera L, Clarke A, Guillarme D, Pell R. 2018. Extending the limits of size exclusion chromatography: Simultaneous separation of free payloads and related species from antibody drug conjugates and their aggregates. *J Chromatogr A.* 1539:19–29.

Gudelj I, Keser T, Vuckovic F, Goreta SS, Pavic T, Dumic J, Primorac D, Lauc G, Gornik O. 2015. Estimation of human age using N-glycan profiles from bloodstains. *Int J Leg Med.* 129:955–961.

Gudelj I, Lauc G, Pezer M. 2018. Immunoglobulin G glycosylation in aging and diseases. *Cell Immunol.* 333:65–79.

Gupta SK, Shukla P. 2018. Glycosylation control technologies for recombinant therapeutic proteins. *Appl Microbiol Biotechnol.* 102:10457–10468.

Halkenscheid L, Bondt A, Scherer HU, Huizinga TW, Wuhrer M, Toes RE. 2018. Coupling carbohydrate interactions are required for high affinity binding between Fc gamma RIII and antibodies lacking core fucose. *Proc Natl Acad Sci USA.* 108:12669–12674.

Haymes JD, Frostell A, Cosgrave EF, Struve WB, Potter O, Davey GP, Karlsson R, Anneren C, Rudd PM. 2014. Fc gamma receptor glycosylation modulates the binding of IgG glycoforms: A requirement for stable antibody interactions. *J Proteome Res.* 13:5471–5483.

Haymes JD, Frostell A, Karlsson R, Muller S, Martin SM, Pauers M, Reuss F, Cosgrave EF, Anneren C, Davey GP et al. 2017. Identification of Fc gamma receptor glycoforms that produce differential binding kinetics for rituximab. *Mol Cell Proteomics.* 16:1770–1788.

Heemskerk AA, Wuhrer M, Busnel JM, Koelmen CA, Selman MH, Vidarsson G, Kapur R, Schoenmaker B, Derks RJ, Deelder AM et al. 2013. Coupling porous sheathless interface MS with transient-ITP in neutral capillaries for improved sensitivity in glycopeptide analysis. *Electrophoresis.* 34:383–387.

Hendrickson OD, Zherdev AV. 2018. Analytical application of lectins. *Crit Rev Anal Chem.* 48:279–292.

Hilliard M, Alley WR Jr, McManus CA, Yu YQ, Hallinan S, Gebler J, Rudd PM. 2017. Glycan characterization of the NIST RM monoclonal antibody using a total analytical solution: From sample preparation to data analysis. *MAbS.* 9:1349–1359.

Hong Q, Ruhaak LR, Stroble C, Parker E, Huang J, Maverakis E, Lebrilla CB. 2015. A method for comprehensive glycosite-mapping and direct quantitation of serum glycoproteins. *J Proteome Res.* 14:5179–5192.

Huffman JE, Pucic-Bakovic M, Klaric L, Henning R, Selman MH, Vuckovic F, Novokmet M, Kristic J, Borowiak M, Muth T et al. 2014. Comparative performance of four methods for high-throughput glycosylation analysis of immunoglobulin G in genetic and epidemiological research. *Mol Cell Proteomics.* 13:1598–1610.

Huhn C, Selman MH, Ruhaak LR, Deelder AM, Wuhrer M. 2009. IgG glycosylation analysis. *Proteomics.* 9:882–913.

Jansen BC, Falck D, de Haan N, Hipgrave Ederven AL, Razzadoro G, Lauc G, Wuhrer M. 2016. LaCyTools: A targeted liquid chromatography-mass spectrometry data processing package for relative quantitation of glycopeptides. *J Proteome Res.* 15:2199–2201.

Joos K, Meckelmann SW, Klein J, Schmitz OJ, Neususs C. 2018. Capillary zone electrophoresis coupled to drift tube ion mobility-mass spectrometry for the analysis of native and APTS-labeled N-glycans. *Anal Bioanal Chem.*

Kammeier GS, Kohler I, Jansen BC, Hensbergen PJ, Mayboroda OA, Falck D, Wuhrer M. 2016. Dopant enriched nitrogen gas combined with sheathless capillary electrophoresis-electrospray ionization-mass spectrometry for improved sensitivity and repeatability in glycopeptide analysis. *Anal Chem.* 88:5849–5856.

Kammeier GSN, Jansen BC, Kohler I, Heemskerk AAM, Mayboroda OA, Hensbergen PJ, Schapper J, Wuhrer M. 2017. Static acid linkage differentiation of glycopeptides using capillary electrophoresis-electrospray ionization-mass spectrometry. *Sci Rep.* 7:3733.

Kapur R, Della Valle L, Sonneveld M, Hipgrave Ederven A, Visser R, Ligthart P, da Haas M, Wuhrer M, van der Schoot CE, Vidarsson G. 2014. Low anti-RhD IgG-Fc-fucosylation in pregnancy: A new variable predicting severity in haemolytic disease of the fetus and newborn. *Br J Haematol.* 166:936–945.

Kemna MJ, Plorom R, van Paassen P, Koelmen CAM, Jansen BC, Damoiseaux J, Cohen Tervaert JW, Wuhrer M. 2017. Galactosylation and sialylation levels of IgG predict relapse in patients with PR3-ANCA associated vasculitis. *EBioMedicine.* 17:108–118.

Keser T, Pavic T, Lauc G, Gornik O. 2018. Comparison of 2-aminobenzamide, procainamide and RapiFluor-MS as derivatizing agents for high-throughput HILIC-UPLC-FLR-MS N-glycan analysis. *Front Chem.* 6:324.

Khatri K, Klein JA, Haserick JR, Leon DR, Costello CE, Metcalf ME, Zaias J. 2017. Microfluidic capillary electrophoresis-mass spectrometry for analysis of monosaccharides, oligosaccharides, and glycopeptides. *Anal Chem.* 89:6645–6655.

Kimzey M, Szabo Z, Sharma V, Gyenes A, Top S, Taylor A, Jones A, Hyche J, Haox T, Vlasenko S. 2015. Development of an instant glycan labeling dye for high throughput analysis by mass spectrometry. *Procyome.* 25:1295–1295.

Kiyoishi M, Czavoever JMM, Tada M, Tamura H, Tanaka T, Terao Y, Morante K, Harazono A, Hashi N, Shibata H et al. 2018. Assessing the heterogeneity of the Fc-glycan of a therapeutic antibody using an engineered Fc gamma Receptor IIIa-immobilized column. *Sci Rep.* 8:3955.

Klapoette SC, Zhang J, Becht S. 2011. Glycosylation characterization of human IgA1 with differential deglycosylation by UPLC-ESI TOF MS. *J Pharm Biomed Anal.* 56:513–520.

Klasic M, Kristic J, Korac P, Horvat V, Markulin D, Vojta A, Reiding KR, Wuhrer M, Lauc G, Zoldos V. 2016. DNA hypomethylation upregulates expression of the MGAT3 gene in HepG2 cells and leads to changes in N-glycosylation of secreted glycoproteins. *Sci Rep.* 6:24363.

Klasic M, Markulin D, Vojta A, Samazjara J, Birus I, Dobrnic P, Venham NT, Trebjesic-Akmacice J, Simurina M, Stambuk J et al. 2018. Promoter methylation of the MGAT3 and BACH2 genes correlates with the composition of the immunoglobulin G glycome in inflammatory bowel disease. *Clin Epigenetics.* 10:75.
Kolarich D, Windwarder M, Alagesan K, Altmann F. 2015. Isomeric-specific analysis of released N-glycans by LC-ESI MS/MS with porous graphitized carbon. *Methods Mol Biol.* 1321:427–435.

Kozak RP, Tortosa CB, Fernandes DL, Spencer DL. 2015. Comparison of proline-238 and aminobenzamide labeling for profiling and identification of glycans by liquid chromatography with fluorescence detection coupled to electrospray ionization-mass spectrometry. *Anal Biochem.* 486:38–40.

Krapp S, Mimura Y, Jefferis R, Huber R, Sondermann P. 2003. Structural analysis of human IgG-Fc glycoforms reveals a correlation between glyco-structure and confounding structure. *J Mol Biol.* 325:979–989.

Kurogochi M, Amano J. 2014. Relative quantitation of glycopeptides based on stable isotope labeling using MALDI-TOF MS. *Molecules.* 19:9944–9961.

Laggeven-Kammeijer GSM, De Haan N, Mohaupt P, Wagt W, Fluis M, Nouta et al. 2019. Sialylation and structural integrity. *J Chromatogr A.* 1511:107860.

Largy E, Cantais F, Van Vyncht G, Beck A, Delobel A. 2017. Orthogonal liquid chromatography-mass spectrometry methods for the comprehensive characterization of therapeutic glycoproteins, from released glycans to intact protein level. *J Chromatogr A.* 1498:128–146.

Lauber MA, Yu YQ, Brousniche DW, Hua Z, Koza SM, Magnelli P, Guthrie E, Taron CH, Fountain KJ. 2015. Rapid preparation of released N-glycans for HILIC analysis using a Labeling reagent that facilitates sensitive fluorescence and ESI-MS detection. *Anal Chem.* 87:5401–5409.

Lau C, Essafi A, Huffman JE, Hayward C, Knezovic A, Katlla JJ, Polasek O, Gornik O, Vitart V, Abrahams GL et al. 2010. Genomics meets glycomics—the first GWAS study of human N-Glycome identifies HNF1alpha as a master regulator of plasma protein fucosylation. *PLoS Genet.* 6:e1001256.

Lau C, Huffman JE, Pacic M, Zgaga L, Adamczyk B, Muzinic A, Novokmet M, Polasek O, Gornik O, Krstic J et al. 2013. Loci associated with N-glycosylation of human immunoglobulin G show pleiotropy with autoimmune diseases and haematological cancers. *PLoS Genet.* 9:e1003225.

Lau C, Vojta A, Zoldos V. 2014. Epigenetic regulation of glycosylation is the quantum mechanics of biology. *Biochim Biophys Acta.* 1840:65–70.

Lau C, Vuckovic F, Bondt A, Pezer M, Wuhrer M. 2018. Trace N-glycans including sulphated species may originate from various plasma glycoproteins and not necessarily IgG. *Nature Genet.* 9:2916.

Leblanc Y, Romainn M, Bioreno N, Chevreux G. 2014. LC-MS analysis of polyclonal IgGs using Idex enzymatic proteolysis for oxidation monitoring. *J Chromatogr B Analyt Technol Biomed Life Sci.* 961:1–4.

Lectenz Biotech. 2019. *Lectenz*® Bio. From: https://lectenz.com/.

Lemmers RFH, Vilaj M, Urda D, Agakof S, Simurina M, Klaric L, Rudan I, Campell H, Hayward C, Wilson JF et al. 2015. Comparison of fluorescent labels for oligosaccharides and introduction of a new postlabeling purification method. *Anal Biochem.* 511:111–117.

Mauko L, Lacher NA, Pelzing M, Nordborg A, Haddad PR, Hilder EF. 2012. Comparison of ZIC-HILIC and graphitized carbon-based analytical approaches combined with exoglycosidase digestions for analysis of glycans from monoclonal antibodies. *J Chromatogr B Analyt Technol Biomed Life Sci.* 911:93–104.

Maxwell EFJ, Ratnapake C, Joyo R, Zhong X, Chen DD. 2011. A promising capillary electrophoresis-electrospray ionization-mass spectrometry method for carbohydrate analysis. *Electrophoresis.* 32:2161–2166.

Mellers SJ, Baenziger JU. 1983. Structures of the O-glycosidically linked oligosaccharides of human IgG. *J Biol Chem.* 258:11557–11563.

Montesino R, Calvo L, Vailin A, Rudd PM, Harvey DJ, Cremata JA. 2012. Structural characterization of N-linked oligosaccharides on mono-
glycomics/proteome initiative multi-institutional study of IgA1. *Mol Cell Proteomics.* 9:719–727.

Walker SH, Lilley LM, Enamorado MF, Comins DL, Muddiman DC. 2011. Hydrophobic derivatization of N-linked glycans for increased ion abundance in electrospray ionization mass spectrometry. *J Am Soc Mass Spectrom.* 22:1309–1317.

Walt D, Aoki-KinoshitaKF, Bertozzi CR, Boons G-J, al. e. 2012. Transforming glycoscience: A roadmap for the future. *Washington (DC).*

Wang JR, Gao WN, Grimm R, Jiang S, Liang Y, Ye H, Li ZG, Yao LF, Huang H, Liu J et al. 2017. A method to identify trace sulfated IgG N-glycans as biomarkers for rheumatoid arthritis. *Nat Commun.* 8:631.

Wang JR, Gao WN, Grimm R, Jiang S, Liang Y, Ye H, Li ZG, Yao LF, Huang H, Liu J et al. 2018. Reply to “Trace N-glycans including sulphated species may originate from various plasma glycoproteins and not necessarily IgG.” *Nat Commun.* 9:2915.

Washburn N, Meccariello R, Duffner J, Getchell K, Holte K, Prod’homme T, Srinivasan K, Prenovitz R, Lansing J, Capila I et al. 2019. Characterization of endogenous human FcgammaRIII by mass spectrometry reveals site, allele and sequence specific glycosylation. *Mol Cell Proteomics.* 18:534–545.

Wohlschlager T, Scheffler K, Forstenlehner IC, Skala W, Senn S, Damoc E, Holzmann J, Huber CG. 2018. Native mass spectrometry combined with enzymatic dissection unravels glycoform heterogeneity of biopharmaceuticals. *Nat Commun.* 9:1713.

Wu G, Hitchen PG, Panico M, North SJ, Barbouche MR, Binet D, Morris HR, Dell A, Hashal SM. 2016. Glycoproteomic studies of IgE from a novel hyper IgE syndrome linked to PGM3 mutation. *Glycoconj J.* 33:447–456.

Wuhrer M, Porcelijn L, Kapur R, Koeleman CA, Deelder AM, de Haas M, Vidarsson G. 2009. Regulated glycosylation patterns of IgG during allotissue response against human platelet antigens. *J Proteome Res.* 8: 450–459.

Yagi H, Takakura D, Roumenina LT, Fridman WH, Sautes-Fridman C, Kawasaki N, Kato K. 2018. Site-specific N-glycosylation analysis of soluble Fcgamma receptor IIIb in human serum. *Sci Rep.* 8:2719.

Yang S, Onigman P, Wu WW, Sjogren J, Nyhlen H, Shen RF, Cipollo J. 2018. Deciphering protein O-glycosylation: Solid-phase chemoenzymatic cleavage and enrichment. *Anal Chem.* 90:8261–8269.

Yang W, Ao M, Hu Y, Li QK, Zhang H. 2018. Mapping the O-glycoproteome using site-specific extraction of O-linked glycopeptides (EXoO). *Mol Syst Biol.* 14:e8486.

Yang Y, Wang G, Song T, Lebrilla CB, Heck AJR. 2017. Resolving the microheterogeneity and structural integrity of monoclonal antibodies by hybrid mass spectrometric approaches. *MAbs.* 9:638–643.

Ye H, Boyne MT 2nd, Buhse LF, Hill J. 2013. Direct approach for qualitative and quantitative characterization of glycoproteins using tandem mass tags and an LTQ Orbitrap XL electron transfer dissociation hybrid mass spectrometer. *Anal Chem.* 85:1531–1539.

Yuan W, Sandy M, Wu J, Koomen J, Goldman R. 2015. Quantitative analysis of immunoglobulin subclasses and subclass specific glycosylation by LC-MS-MRM in liver disease. *J Proteomics.* 116:24–33.

Zauner G, Koeleman CA, Deelder AM, Wuhrer M. 2010. Protein glycosylation analysis by HILIC-LC-MS of proteinase K-generated N- and O-glycopeptides. *J Sep Sci.* 33:903–910.

Zauner G, Selman MH, Bondt A, Rombouts Y, Blank D, Deelder AM, Wuhrer M. 2013. Glycoproteomic analysis of antibodies. *Mol Cell Proteomics.* 12:856–865.

Zhang L, Luo S, Zhang B. 2016. The use of lectin microarray for assessing glycosylation of therapeutic proteins. *MAbs.* 8:524–533.

Zhao J, Li S, Li C, Wu SL, Xu W, Chen Y, Shameem M, Richardson D, Li H. 2016. Identification of low abundant isomeric N-glycan structures in biological therapeutics by LC/MS. *Anal Chem.* 88:7049–7059.

Zhong X, Chen Z, Snovida S, Liu Y, Rogers JC, Li L. 2015. Capillary electrophoresis-electrospray ionization-mass spectrometry for quantitative analysis of glycans Labeled with multiplex carbonyl-reactive tandem mass tags. *Anal Chem.* 87:6527–6534.

Zhou S, Veillon L, Dong X, Huang Y, Mechref Y. 2017. Direct comparison of derivatization strategies for LC-MS/MS analysis of N-glycans. *Analyst.* 142:4446–4455.