



Short review (expert opinion)

N-glycosylation heterogeneity and the influence on structure, function and pharmacokinetics of monoclonal antibodies and Fc fusion proteins

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ABSTRACT

Monoclonal antibody and Fc fusion protein drugs are complex heterogeneous mixtures of numerous different protein variants and modifications. N-glycosylation as one of the most complex post-translational modification influences the structural characteristics of the antibodies Fc part thereby potentially modulating effector function and pharmacokinetics. Several investigations on the relationship between N-glycosylation and pharmacokinetics have been published. However, this structure–function relationship is not fully understood. In this review potential alterations with focus on N-glycosylation of mAbs and Fc fusion proteins and the possible effects on the pharmacokinetics are reviewed and the current understandings of the underlying mechanisms are described.

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1. Introduction

The fusion of murine myeloma cells with B-cells was a groundbreaking experiment of Köhler and Milstein and made production of antibodies in cell culture possible [1]. It was the beginning of immunoassays and therapeutic antibodies. In 2012, more than 35 years later, 34 recombinant monoclonal antibodies (mAbs) were marketed in the EU and US, mostly produced in CHO and SP2/0 cells [2]. The vast majority of marketed mAbs belongs to the IgG class or more precisely to the subclasses IgG1 and to some extent IgG2 and IgG4. Two-thirds of the marketed mAbs are either human or humanized and a small percentage is chimeric or murine [2]. With more than 1300 amino acids resulting in a mass of approximately 150 kDa mAbs are large molecules built from two heavy chains with 50 kDa each and two light chains with 25 kDa each (Fig. 1). The different heavy chains (γ 1, γ 2, γ 3 and γ 4) divide the IgGs into their subclasses 1–4. The light chains are the κ -type and λ -type. Heavy and light chains are connected by disulfide bridges giving the antibody its Y-shaped structure (Fig. 1). Intra-chain disulfide bridges further stabilize the folding, 16 disulfide bridges per IgG1 and 7 per chain. The heavy and light chains consist of different domains. The variable domains variable light (VL)

and variable heavy (VH) contain hypervariable regions that are responsible for antigen binding. The remaining domains are conserved sequences named constant domains constant light (CL) and constant heavy (CH1–3). The CH2 domain of each heavy chain contains one N-glycosylation site at approximately Asn297 and about one-fifth of human IgGs carry a N-glycosylation motif in the variable region [3,4]. The size and structure of IgGs give rise to a large number of possible alterations and modifications (Table 1) turning IgG drugs and in conclusion recombinant mAb drugs to heterogenic protein mixtures. Several of these alterations can have tremendous influence on the structure, PK and function of monoclonal antibodies. The complex post-translational modification of antibodies, N-glycosylation, will be discussed in detail in the following.

2. N-glycosylation and its influence on mAb structure and mAb effector function

Like most extracellular glycoproteins, therapeutic proteins and specifically also mAbs undergo glycosylation in the ER and Golgi network of cells. The glycan structures of therapeutic mAbs can be of importance for the efficacy and safety of the drug [21]. Monoclonal antibodies have one conserved N-linked glycosylation site at the Fc part at position N297. Approximately 20% contain a second N-linked glycosylation site in their variable region. Both sites are

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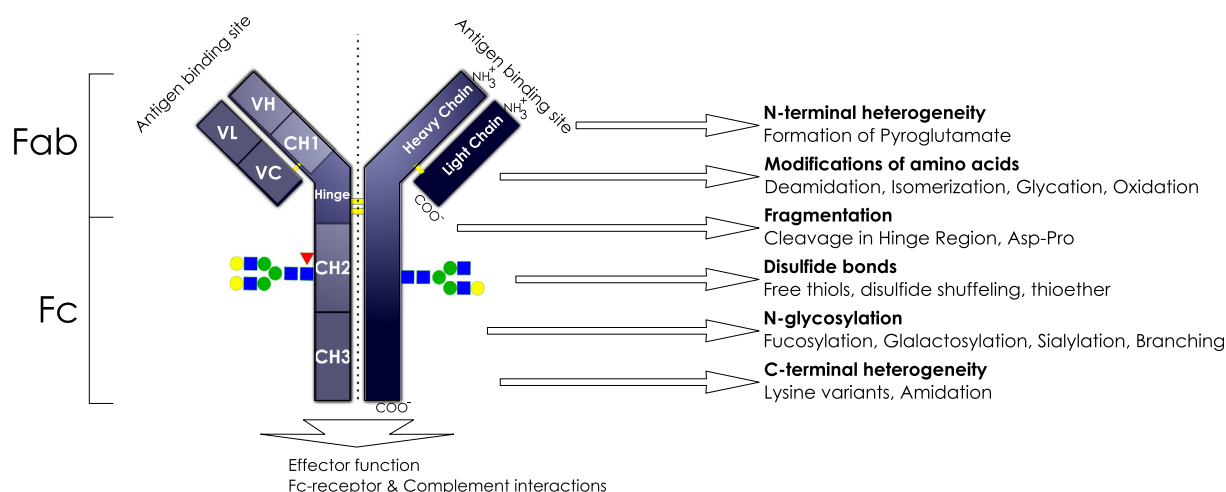


Fig. 1. Schematic structure of an IgG1 molecule. An IgG consists of two heavy and two light chains that contain several domains. The variable domains variable light (VL) and variable heavy (VH) that form the antigen binding site and the constant domains CL (constant light) and CH1–3 (constant heavy) building the framework. The IgG can be furthermore divided into the Fab (fragment antigen binding) and Fc (fragment crystallizable) which induce the effector functions. On the right possible modifications and alterations of the IgG are listed.

Table 1
Common modifications of biopharmaceuticals and their impact on structure and function.

Modification	Effect	Reference(s)
Glu or Gln cyclization at N-terminus	Loss of positive N-terminal charge	[5–7]
Lys variants at C-terminus	Introduction of a positive charge	[7,8]
Deamidation (Asn to Asp)	Introduction of a negative charge (Asn to Asp)	[5,7,9–11]
Isomerization (Asp to isoAsp)	Introduction of an additional CH ₂ group into the protein backbone (Asp to iAsp)	[5,7,12]
Deamidation (Gln to Glu)	Introduction of a negative charge	[13,14]
Glycation	Loss of a positive charge	[7,15,16]
Oxidation (Cys, His, Met, Tyr, Trp)	Increase of polarity	[5,7]
Disulfide bond heterogeneity (Shuffling, thioether and trisulfide formation)	Changes in the protein conformation	[17–20]
Glycosylation (N-linked, O-linked)	Influence on the structure and function	See next section

located on the heavy chain [3]. Glycosylation of biopharmaceuticals shows a high grade of heterogeneity and N-glycans belong to the most complex and diverse structures in nature due to the high number of different sugar moieties and the multitude of possible linkages. Fig. 2 shows the three different N-glycan types high mannose, complex and hybrid that are found on IgGs with their respective linkage. Complex and hybrid types exist with core fucosylation, addition of a fucose residue to the innermost N-acetylglucosamine, and without core fucosylation. mAbs represent a special group of glycoproteins as their N-glycans are of limited size. mAbs are usually free of N-glycans with more than two antennae and furthermore the sialic acid content is low compared with other glycoproteins [22]. This circumstance can be explained by the fact that the glycosylation site in the CH₂ domain at Asn297 is buried in the protein structure. Typically, antibodies contain a high percentage of complex bi-antennary glycans with core-fucosylation [22–24].

N-glycans have important structural functions. They stabilize the CH₂ domain of IgGs and deglycosylation makes mAbs thermally less stable and more susceptible to unfolding. In addition deglycosylated mAbs are more prone to aggregation [25]. Not only thermal and colloidal stability, but also functionality of the IgG is influenced by the attached N-glycans and their size [26]. In addition to the stabilization of the CH₂ domain the attached N-glycans greatly influence the folding of the Fc part. Krapp et al. investigated crystallized Fc parts of IgG molecules with different homogenous glycosylations and could demonstrate that the

conformation of the CH₂ domain depends on the attached N-glycans [27]. Larger N-glycans, e.g. bi-antennary complex type with terminal galactosylation, open up the Fc part in the CH₂ region to a horseshoe like structure, whereas smaller attached N-glycans like the core structure favor a more “closed” Fc conformation. This open and closed formation can greatly influence the effector functions induced by interactions of the Fc part with Fc receptor molecules. The fact that crystallization of deglycosylated IgG was not possible due to the high flexibility of the CH₂ domain shows the importance of N-glycan protein interactions [27]. Hydrogen/Deuterium exchange MS experiments resulted in similar findings. Houde et al. showed that terminal galactosylation has major impact on the conformation of the Fc part and that fucosylation alone does not impact the conformation [28]. NMR analysis of G2F glycosylated mAbs revealed that the terminal galactose residues are exposed and accessible for protein binding and that there are differences between the 1,3 and the 1,6 arm concerning flexibility and accessibility [29]. These findings led to the assumption that Fc N-glycosylation influences the effector function of proteins interacting with the CH₂ domain. Upon antigen binding mAbs are able to induce effector functions mediated by their Fc-part. By binding to Fc-receptors or complement proteins mAbs induce antibody-dependent cell-mediated cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC), respectively. ADCC is induced after binding of Fc-γ receptors (FcγR) to the Fc part [30]. The affinity of FcγRs to the Fc part is influenced by N-glycosylation in the CH₂ domain [28,31–34]. As a consequence

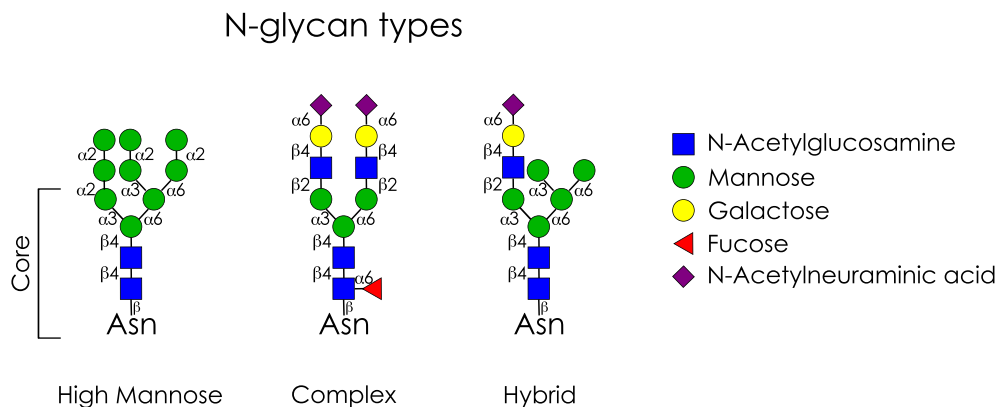


Fig. 2. Types of N-glycans. The three different types (High Mannose, Complex and Hybrid) share a common core structure including the first two N-acetylglucosamine residues and the first three mannose residues.

ADCC is enhanced for IgGs with lower fucosylation [31,35]. Binding affinity of the complement protein C1q to the IgG Fc which is involved in CDC is also influenced by N-glycosylation and an increasing content of terminal galactose enhances CDC [35]. By influencing CDC and ADCC, two important effector functions of therapeutic mAbs, N-glycosylation was thought to also have an impact on the pharmacokinetics of the glycosylated biopharmaceuticals.

3. N-glycosylation and its effect on pharmacokinetics of mAbs

Monoclonal antibodies are large and complex structures with a different behavior in terms of their pharmacology and pharmacokinetics (PK) compared to low molecular weight drugs. Monoclonal antibodies show dose-dependent maximal plasma concentrations in the range of $\mu\text{g/ml}$ (nM range). mAbs typically exhibit a body half-life between 10 and 25 days, since they are not eliminated through kidney filtration due to their size and additionally escape fast degradation in the lysosomes through the neonatal Fc receptor (FcRn) recycling mechanism [36,37]. mAbs are usually administered parenterally by subcutaneous (sc), intramuscular (im) or intravenous (iv) injection. Whereas iv bioavailability is 100% per definition, for sc and im administration bioavailability values of 50–100% are mostly reported [36].

Already in the 1970s, first evidence for the influence of sialic acids on the pharmacokinetics of glycoproteins was reported. Morrell et al. observed that desialylated plasma proteins were cleared faster from circulation after injection into rats [38]. There are two known major pathways for selective glycoprotein clearance. Glycoproteins in circulation with accessible terminal galactosylation are bound and cleared by the asialoglycoprotein receptor expressed in the liver [39,40]. The second way of selective clearance of glycoproteins is executed by the mannose receptor which is most markedly expressed on immune cells [41–43]. The mannose receptor binds selectively to mannose and N-acetylglucosamine residues of N-glycans [41,42]. The enzymatic conversion of glycans *in vivo* is the third option explaining the selective removal of distinct glycoforms [44–46]. There are two basic options to investigate the effect of different glyco-variants on protein pharmacokinetics. Either distinct glyco-variants are enriched and compared in several study groups or the relative composition of different glyco-variants is directly followed in one study group. Millward et al. studied a mAb enriched to a high mannose portion at the Fc part of approximately 50% and a second mAb enriched for glycosylation in the Fab region. The PK profiles of the different mAb preparations were compared in mice by ELISA and

the glycan pattern was analyzed at several time points by HPLC. No significant differences in PK properties were found [47]. A second investigation in mice which compared PK parameters of degalactosylated IgGs with non-modified IgG came to the conclusion that degalactosylated IgG with terminal GlcNAc has a significantly longer half-life [48]. Production and characterization of three differently glycosylated mAb qualities (Hybrid (+/– F), Complex (+/– F) and high mannose) against CD20 with bioassays (CDC, ADCC) and an *in vivo* study in mice demonstrated that the complex glycosylated mAb had a longer half-life, which is independent from fucosylation. Hybrid glycosylated mAb had a slightly shorter half-life and high mannose glycosylated mAb showed a strong decrease in half-life [49]. On summarizing, the results from mice glycan PK profiling render an unclear picture with two studies reaching contradictory conclusions concerning the influence of high mannose structures on mAb PK in mice [47,49].

Analysis of affinity purified mAb (IgG2) from clinical samples of two healthy human test subjects showed that the relative contribution of high mannose species M6–M9 to the total mAb plasma concentration decreased over time whereas M5 levels increased by about the same percentage. It was hypothesized that the reduction of the IgG2 high mannose glycan blood levels is due to glycosidase activity in serum converting M6–M9 into M5 and not due to faster clearance. This hypothesis was supported with results from an *in vitro* assay. Incubation of the IgG2 in serum led to the same high mannose conversion as observed in the case study [44]. The same group later published a second glycan PK profiling by mass spectrometry approach based on glycopeptide analysis by MS [45]. Analysis of samples from four human subjects after administration of either one IgG1 or one of three IgG2 molecules resulted in the finding that high mannose N-glycan M5 is selectively cleared from serum [45]. The authors furthermore showed that the IgG2 M5:M5 glycoform, the pairing of two M5 glycosylated heavy chains during protein biosynthesis is favored. A similar investigation of an IgG1 biopharmaceutical in humans confirmed the previously published [44] conversion of high mannose glycans *in vitro*. The results of a clinical study including 15 healthy volunteers published in combination with the *in vitro* study showed that M5 had an increased serum clearance whereas M6 and M7 decreased in the first 6 h after administration followed by decreasing M5 levels and constant M6 and M7 portions (Fig. 3) [46]. We have recently reported that this high mannose conversion and selective clearance of M5 are similar in rabbits after subcutaneous administration of an IgG1 [50]. The production of mAbs with one distinct N-glycan and administration to mice demonstrated that a mAb with only M5 or M8/9 has an increased clearance and a shorter half-life. Furthermore, it was highlighted that the glycan pattern of the mAb with

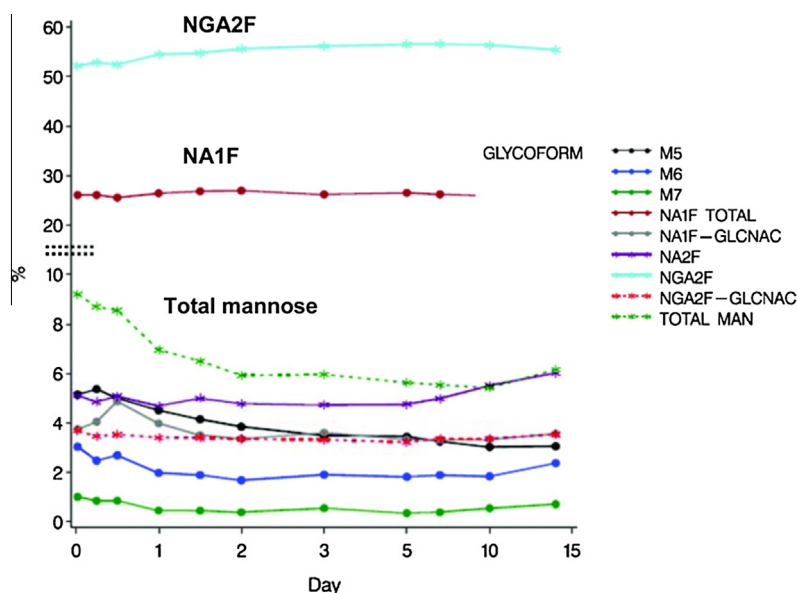


Fig. 3. Mean percentage of glycan species of mAb-1 in serum following single dose administration in healthy individuals. From Alessandri et al. *mAbs* 2012, 4, 509–520 [46].

M8/9 changed over time to a high percentage of M6. This observation could be reproduced *in vitro* by incubation of the mAb in mice serum [51]. Conversion of high mannose glycans to M6 instead of M5 reported for mice implies a different underlying mechanism. A mouse study comparing yeast N-glycosylation (yeast specific high mannose type) and human glycosylation (complex type) revealed that antibodies carrying yeast glycosylation have shorter serum half-life than antibodies with human glycosylation [52]. The influence of the glycosylation of the variable domain on antibody clearance was investigated by different groups [47,53]. As already mentioned Millward et al. did not find any influence of the variable domain N-glycosylation on the PK. In contrast Huang et al. came to the conclusion that molecules with bi-antennary glycans lacking galactosylation (terminal GlcNAc) attached in the variable domain are cleared slightly faster from circulation [53].

4. N-glycosylation and its effect on pharmacokinetics of Fc fusion proteins

Fc part containing fusion proteins are closely related to mAbs, because of the effector function induced by the Fc part of both biopharmaceuticals. Fc fusion proteins often carry several N-glycosylation sites in the non-IgG fusion protein part [54–56]. There are a few reports on an influence of the terminal glycan residues of Fc fusion proteins on PK. Keck et al. compared Fc fusion protein batches with different N-glycosylation pattern, specifically terminal GlcNAc, galactosylation and sialylation [56]. However, no site specific N-glycan analysis was performed which would enable to link effects to the Fc or the fusion protein part. The authors found that terminal GlcNAc was cleared faster. It was hypothesized that the mannose receptor might be responsible for the clearance as the 3D structure of the receptors demonstrated that the receptor can bind terminal GlcNAc containing glycans [56]. In accordance with these findings Jones et al. represented that N-glycans with terminal GlcNAc residues have a higher clearance rate. Their protein of interest was the fusion protein lenercept containing a Fc part and two extracellular domains of a TNF-alpha receptor. Sialic acids and terminal galactosylation revealed only minor to no impact on the PK [55]. Kogelberg et al. postulated a clearance mechanism for highly mannosylated proteins after studying an antibody Fc–enzyme fusion protein produced in the yeast *Pichia*

pastoris. They provided evidence for clearance of the glycoprotein by the mannose receptor, a glycan specific receptor which is expressed beside others in sinusoidal endothelial cells in the liver [57]. Reports from Liu et al. comparing differently glycosylated fusion proteins and mAbs produced in either glyco-engineered *P. pastoris* or CHO cells highlighted that the sialic acid content of fusion proteins modulates the pharmacokinetics [52,58]. Lower levels of sialic acids correlated with decreased serum half-life.

5. Analysis of N-glycosylation in pharmacokinetic studies

Immunoassays became the reference technique for PK-studies of therapeutic proteins, especially ELISA where the protein is captured by a specific antibody or in the case of therapeutic antibodies also by an anti-idiotypic antibody, an anti-species antibody or the appropriate antigen and finally detected by an enzyme-linked secondary antibody [59]. However structural changes such as deamidation, oxidation and especially the heterogeneity of glycoforms of the therapeutic protein, as discussed in previous sections, could alter the pharmacokinetic properties [60,61] and immunoassay techniques are not able to detect such types of differences [59,62]. ELISA simultaneously quantifies all protein variants delivering the average PK profile of all variants and cannot distinguish between glyco-variants. Hence different analytical techniques are necessary. For the investigation of individual N-glycans LC and MS based approaches were mostly used in the literature [44–47,49,53–55]. Beside the analysis of intact glycoproteins which provides information about the most abundant glycoforms, the analysis of glycopeptides and released N-glycans is frequently used. Enzymatic release of N-glycans following labeling with a fluorophore like anthranilamide or anthranilic acid and analysis by LC or LC-MS is perhaps the most widespread approach used in industry and academia [23,24,63–69]. More sensitive approaches such as nano-LC-MS, CE and CE-MS with LIF detection are also common and suitable for glycan related PK investigations [70–77]. The advantage of these technologies for the glycan PK profiling is the possibility to analyze each N-glycan individually from a complex mixture which in turn leads to more accurate results compared to e.g. studies employing immunoassays after enrichment of specific glycoforms.

6. Glyco-engineering of biopharmaceuticals

As summarized in the previous sections there is strong evidence in the literature for the influence of N-glycosylation on structure, function and pharmacokinetics of monoclonal antibodies and Fc fusion proteins. Consequently, glyco-engineering, the controlled biosynthesis of only distinct glycan structures became of high interest in the biopharmaceutical industry. There are multiple strategies to produce biotechnology products with defined N-linked glycans [21,78]. One straightforward yet highly sophisticated approach to control the N-glycosylation of recombinant antibodies is based on special in-process controls [79]. Here no manipulation of the genome or set of enzymes is required; instead a tight control of temperature, pH and feed is used to influence the glycan species (e.g. high mannose and terminal galactosylation levels). Another option to influence the glycan pattern is the use of specific inhibitors of glycosyltransferases. Rillahan et al. demonstrated that the addition of fluorinated monosaccharide analogs efficiently inhibited the corresponding fucosyl or sialyltransferases [80]. Another group used RNAi constructs to knock down α -1,3-fucosyltransferase and β -1,2-xylosyltransferase which allowed expression of a mAb with human N-glycans in the aquatic plant *Lemna minor*. The produced mAb had a homogeneous glycan pattern and better ADCC and effector function [81]. Humanization of N-glycans in the yeast *P. pastoris* was also reported. By introducing a set of human enzymes to the yeast genome it was possible to express biopharmaceuticals with complex N-glycans instead of the yeast high mannose glycans [82]. The reduction of the fucosylation levels has been of central interest since the absence of this single monosaccharide causes a drastic increase in efficacy. For a permanent absence of fucose the generation of knockout cell lines is required which can be achieved by either silencing or removing the Fucosyltransferase 8 (FUT8) or a GDP-mannose 4,6-dehydratase (GMD) gene [83,84]. Another approach to lower the fucosylation is the GlycArt technology, which renders afucosylated antibodies with a high content of bisecting glycans by overexpression of a distinct glycosyltransferase and change of the localization domain [85]. It was shown that only the absence of the core fucose was responsible for the enhanced ADCC, but not the presence of the bisecting N-glycans [86]. This technology was utilized to generate two antibodies with high ADCC in the oncology area, GA101 (obinutuzumab; approved) targeting CD20 and GA201 targeting EGFR [87,88]. Another approved antibody with improved ADCC is mogamulizumab also based on a afucosylation technology [89].

7. Conclusion and future perspectives

Monoclonal antibody drugs are complex and heterogeneous mixtures of protein variants. The N-glycosylation on the antibody structure can influence stability, function and pharmacokinetics. In some publications distinct glycosylation fractions were enriched and studied. However, with this approach a new artificial glycosylation pattern is generated that does not necessarily reflect the normal N-glycosylation of the biologic of interest and may render different outcome especially if other protein variants with an influence were co-enriched. The benefit of this approach is that simple standard analytics e.g. utilizing immunoassays can be performed to characterize the PK. Individual N-glycans analysis by MS based approaches can be used to analyze the heterogeneous biopharmaceutical directly. The major advantage of this MS approach is that the N-glycan levels can be analyzed in a relative manner. Individual N-glycans with influence on the PK can be identified rather simply. However, the MS approach requires high sensitivity. The studies investigating the relationship between N-glycosylation

and pharmacokinetics led to in part contradictory findings. Overall it is conclusive that high mannose glycans influence the PK of IgGs by increasing the clearance rate through the mannose receptor. For other N-glycans and glycoforms the results are less clear. Two studies investigating IgG variable domain glycosylation rendered inconsistent results and another study comparing differently glycosylated enriched fractions reported no difference. These inconsistent findings may be due to differences in study set-up (e.g. enrichment of distinct glycoforms vs. simultaneous quantification using LC-MS based methods), species (mouse, human), IgG subclass and IgG source (human, humanized, etc.). Results for the more complex glycosylated Fc fusion proteins with additional glycosylation sites in the fusion protein part also show an unclear picture for the effect of N-glycosylation on the pharmacokinetics. The N-glycans of the fusion proteins were mostly analyzed without site specificity. The N-glycans at the Fc part are buried between the heavy chains whereas the receptor part N-glycans could be more accessible for the interaction with specific enzymes or receptors. This phenomenon could result in different impact on the pharmacokinetics. Thus, over the last decade several investigations on the relationship between N-glycosylation of therapeutic proteins and pharmacokinetics were performed. With the use of sensitive technologies such as LC-MS it became possible to change the analysis from comparing fractions of biopharmaceuticals enriched for specific N-glycans to the direct analysis of the complex glycosylated biopharmaceutical of interest. This resulted in more reproducible findings. The development of glyco-engineering technologies enabled the successful production and approval of more homogeneously glycosylated antibodies. Especially for oncology products the removal of the core fucose and the resulting higher ADCC was a success. However, this complete removal of fucose cannot always be used, as e.g. in anti-inflammatory diseases ADCC is not a desired effector function. Here, complete fucosylation is favorable. In addition, the production process itself has a considerable influence on the resulting N-glycan pattern. Glyco-engineering of multiple glycosylated fusion proteins can be even more difficult if a certain glycan is for example involved in target binding. With the rising number of new formats of biopharmaceuticals and biosimilars in development the need for N-glycan PK profiling and comparative studies will rise as well as the need for further glyco-engineering and optimization.

Disclosures

Fabian Higel and Andreas Seidl are both employees of HEXAL AG and therefore receive salaries. HEXAL AG funded the drafting, review and approval of the manuscript.

References

- [1] G. Köhler, C. Milstein, Continuous cultures of fused cells secreting antibody of predefined specificity, *Nature* 256 (1975) 495–497.
- [2] J.M. Reichert, Marketed therapeutic antibodies compendium, *MABs* 4 (2012) 413–415, <http://dx.doi.org/10.4161/mabs.19931>.
- [3] C.A. Abel, H.L. Spiegelberg, H.M. Grey, Carbohydrate content of fragments and polypeptide chains of human, gamma. G-myeloma proteins of different heavy-chain subclasses, *Biochemistry* 7 (1968) 1271–1278, <http://dx.doi.org/10.1021/bi00844a004>.
- [4] F. Matsuda, K. Ishii, P. Bourvagnet, K.I. Kuma, H. Hayashida, T. Miyata, et al., The complete nucleotide sequence of the human immunoglobulin heavy chain variable region locus, *J. Exp. Med.* 188 (1998) 2151–2162.
- [5] W.E.I. Wang, S. Singh, D.L. Zeng, K. King, S. Nema, W.E.T. Al, Antibody structure, instability, and formulation, *J. Pharm. Sci.* 96 (2007) 1–26, <http://dx.doi.org/10.1002/jps>.
- [6] Y.D. Liu, A.M. Goetze, R.B. Bass, G.C. Flynn, N-terminal glutamate to pyroglutamate conversion in vivo for human IgG2 antibodies, *J. Biol. Chem.* 286 (2011) 11211–11217, <http://dx.doi.org/10.1074/jbc.M110.185041>.
- [7] H. Liu, G. Gaza-Bulseco, D. Faldu, C. Chumsae, J. Sun, Heterogeneity of monoclonal antibodies, *J. Pharm. Sci.* 97 (2008) 2426–2447, <http://dx.doi.org/10.1002/jps.21180>.

- [8] B. Cai, H. Pan, G.C. Flynn, C-terminal lysine processing of human immunoglobulin G2 heavy chain in vivo, *Biotechnol. Bioeng.* 108 (2011) 404–412, <http://dx.doi.org/10.1002/bit.22933>.
- [9] D. Chelius, D.S. Rehder, P.V. Bondarenko, Identification and characterization of deamidation sites in the conserved regions of human immunoglobulin gamma antibodies, *Anal. Chem.* 77 (2005) 6004–6011, <http://dx.doi.org/10.1021/ac050672d>.
- [10] L.W. Dick, D. Qiu, R.B. Wong, K.-C. Cheng, Isomerization in the CDR2 of a monoclonal antibody: binding analysis and factors that influence the isomerization rate, *Biotechnol. Bioeng.* 105 (2010) 515–523, <http://dx.doi.org/10.1002/bit.22561>.
- [11] R.J. Harris, B. Kabakoff, F.D. Macchi, F.J. Shen, M. Kwong, J.D. Andya, et al., Identification of multiple sources of charge heterogeneity in a recombinant antibody, *J. Chromatogr. B Biomed. Sci. Appl.* 752 (2001) 233–245.
- [12] D.W. Aswad, M.V. Paranandi, B.T. Schurter, Isoaspartate in peptides and proteins: formation, significance, and analysis, *J. Pharm. Biomed. Anal.* 21 (2000) 1129–1136, [http://dx.doi.org/10.1016/S0731-7085\(99\)00230-7](http://dx.doi.org/10.1016/S0731-7085(99)00230-7).
- [13] H. Liu, G. Gaza-bulseco, C. Chumsae, Glutamine deamidation of a recombinant monoclonal antibody, *Rapid Commun. Mass Spectrom.* (2008) 4081–4088, <http://dx.doi.org/10.1002/rcm>.
- [14] R. Bischoff, H.V.J. Kolbe, Deamidation of asparagine and glutamine residues in proteins and peptides: structural determinants and analytical methodology, *J. Chromatogr. B Biomed. Sci. Appl.* 662 (1994) 261–278, [http://dx.doi.org/10.1016/0378-4347\(94\)00203-7](http://dx.doi.org/10.1016/0378-4347(94)00203-7).
- [15] A.M. Goetze, Y.D. Liu, T. Arroll, L. Chu, G.C. Flynn, Rates and impact of human antibody glycation in vivo, *Glycobiology* 22 (2011) 221–234, <http://dx.doi.org/10.1093/glycob/cwr141>.
- [16] J. Yang, R. Primack, M. Frohn, W. Wang, P. Luan, M.W. Retter, et al., Impact of glycation on antibody clearance, *AAPS J.* 17 (2015) 237–244, <http://dx.doi.org/10.1208/s12248-014-9694-4>.
- [17] H. Liu, K. May, Disulfide bond structures of IgG molecules: structural variations, chemical modifications and possible impacts to stability and biological function, *MAbs* 4 (2012) 17–23, <http://dx.doi.org/10.4161/mabs.4.1.18347>.
- [18] T.M. Dillon, M.S. Ricci, C. Vezina, G.C. Flynn, Y.D. Liu, D.S. Rehder, et al., Structural and functional characterization of disulfide isoforms of the human IgG2 subclass – supplementary material, *J. Biol. Chem.* 283 (2008) 1–15, <http://dx.doi.org/10.1074/jbc.M709988200>.
- [19] A. Guo, M. Han, T. Martinez, R.R. Ketchem, S. Novick, C. Jochheim, et al., Electrophoretic evidence for the presence of structural isoforms specific for the IgG2 isotype, *Electrophoresis* 29 (2008) 2550–2556, <http://dx.doi.org/10.1002/elps.200800083>.
- [20] J. Wypych, M. Li, A. Guo, Z. Zhang, T. Martinez, M.J. Allen, et al., Human IgG2 antibodies display disulfide-mediated structural isoforms, *J. Biol. Chem.* 283 (2008) 16194–16205, <http://dx.doi.org/10.1074/jbc.M709987200>.
- [21] R. Jefferis, Glycosylation as a strategy to improve antibody-based therapeutics, *Nat. Rev. Drug Discov.* 8 (2009) 226–234, <http://dx.doi.org/10.1038/nrd2804>.
- [22] G.C. Flynn, X. Chen, Y.D. Liu, B. Shah, Z. Zhang, Naturally occurring glycan forms of human immunoglobulins G1 and G2, *Mol. Immunol.* 47 (2010) 2074–2082, <http://dx.doi.org/10.1016/j.molimm.2010.04.006>.
- [23] F. Higel, U. Demelbauer, A. Seidl, W. Friess, F. Sörgel, Reversed-phase liquid-chromatographic mass spectrometric N-glycan analysis of biopharmaceuticals, *Anal. Bioanal. Chem.* 405 (2013) 2481–2493, <http://dx.doi.org/10.1007/s00216-012-6690-3>.
- [24] X. Chen, G.C. Flynn, Analysis of N-glycans from recombinant immunoglobulin G by on-line reversed-phase high-performance liquid chromatography/mass spectrometry, *Anal. Biochem.* 370 (2007) 147–161, <http://dx.doi.org/10.1016/j.ab.2007.08.012>.
- [25] K. Zheng, C. Bantog, R. Bayer, The impact of glycosylation on monoclonal antibody conformation and stability, *MAbs* 3 (2011) 568–576, <http://dx.doi.org/10.4161/mabs.3.6.17922>.
- [26] Y. Mimura, S. Church, R. Ghirlando, P.R. Ashton, S. Dong, M. Goodall, et al., The influence of glycosylation on the thermal stability and effector function expression of human IgG1-Fc: properties of a series of truncated glycoforms, *Mol. Immunol.* 37 (2001) 697–706.
- [27] S. Krapp, Y. Mimura, R. Jefferis, R. Huber, P. Sondermann, Structural analysis of human IgG-Fc glycoforms reveals a correlation between glycosylation and structural integrity, *J. Mol. Biol.* 325 (2003) 979–989, [http://dx.doi.org/10.1016/S0022-2836\(02\)01250-0](http://dx.doi.org/10.1016/S0022-2836(02)01250-0).
- [28] D. Houde, Y. Peng, S.A. Berkowitz, J.R. Engen, Post-translational modifications differentially affect IgG1 conformation and receptor binding, *Mol. Cell. Proteomics* 9 (2010) 1716–1728, <http://dx.doi.org/10.1074/mcp.M900540-MCP200>.
- [29] A.W. Barb, J.H. Prestegard, NMR analysis demonstrates immunoglobulin G N-glycans are accessible and dynamic, *Nat. Chem. Biol.* 7 (2011) 147–153, <http://dx.doi.org/10.1038/nchembio.511>.
- [30] S. Radaev, P. Sun, Recognition of immunoglobulins by Fcγ receptors, *Mol. Immunol.* 38 (2002) 1073–1083.
- [31] R. Abès, J.-L. Teillaud, Impact of glycosylation on effector functions of therapeutic IgG, *Pharmaceuticals* 3 (2010) 146–158, <http://dx.doi.org/10.3390/ph3010146>.
- [32] G. Zou, H. Ochiai, W. Huang, Q. Yang, C. Li, L. Wang, Chemoenzymatic synthesis and Fcγ receptor binding of homogeneous glycoforms of antibody Fc domain. Presence of a bisecting sugar moiety enhances the affinity of Fc to FcγIIIa receptor, *J. Am. Chem. Soc.* 133 (2011) 18975–18991, <http://dx.doi.org/10.1021/ja208390n>.
- [33] Y. Mimura, P. Sondermann, R. Ghirlando, J. Lund, S.P. Young, M. Goodall, et al., Role of oligosaccharide residues of IgG1-Fc in Fc gamma R1b binding, *J. Biol. Chem.* 276 (2001) 45539–45547, <http://dx.doi.org/10.1074/jbc.M107478200>.
- [34] M. Satoh, S. Iida, K. Shitara, Non-fucosylated therapeutic antibodies as next-generation therapeutic antibodies, *Expert Opin. Biol. Ther.* 6 (2006) 1161–1173, <http://dx.doi.org/10.1517/14712598.6.11.1161>.
- [35] T.S. Raju, Terminal sugars of Fc glycans influence antibody effector functions of IgGs, *Curr. Opin. Immunol.* 20 (2008) 471–478, <http://dx.doi.org/10.1016/j.coi.2008.06.007>.
- [36] E.D. Lobo, R.J. Hansen, J.P. Balthasar, Antibody pharmacokinetics and pharmacodynamics, *J. Pharm. Sci.* 93 (2004) 2645–2668, <http://dx.doi.org/10.1002/jps.20178>.
- [37] D.C. Roopenian, S. Akilesh, FcRn: the neonatal Fc receptor comes of age, *Nat. Rev. Immunol.* 7 (2007) 715–725, <http://dx.doi.org/10.1038/nri2155>.
- [38] A.G. Morell, G. Gregoriadis, I.H. Scheinberg, J. Hickman, G. Ashwell, The role of sialic acid in determining the survival of glycoproteins in the circulation, *J. Biol. Chem.* 246 (1971) 1461–1467.
- [39] R.J. Stockert, The asialoglycoprotein receptor: relationships between structure, function, and expression, *Physiol. Rev.* 75 (1995) 591–609.
- [40] G. Ashwell, J. Harford, Carbohydrate-specific receptors of the liver, *Annu. Rev. Biochem.* 51 (1982) 531–554, <http://dx.doi.org/10.1146/annurev.bi.51.070182.002531>.
- [41] P. Allavena, M. Chieppa, P. Monti, L. Piemonti, From pattern recognition receptor to regulator of homeostasis: the double-faced macrophage mannose receptor, *Crit. Rev. Immunol.* 24 (2004) 179–192, <http://dx.doi.org/10.1615/CritRevImmunol.v24.i3.20>.
- [42] S.J. Lee, S. Evers, D. Roeder, A.F. Parlow, J. Risteli, L. Risteli, et al., Mannose receptor-mediated regulation of serum glycoprotein homeostasis, *Science* 295 (2002) 1898–1901, <http://dx.doi.org/10.1126/science.1069540>.
- [43] P.R. Taylor, S. Gordon, L. Martinez-Pomares, The mannose receptor: linking homeostasis and immunity through sugar recognition, *Trends Immunol.* 26 (2005) 104–110, <http://dx.doi.org/10.1016/j.it.2004.12.001>.
- [44] X. Chen, Y.D. Liu, G.C. Flynn, The effect of Fc glycan forms on human IgG2 antibody clearance in humans, *Glycobiology* 19 (2009) 240–249, <http://dx.doi.org/10.1093/glycob/cwn120>.
- [45] A.M. Goetze, Y.D. Liu, Z. Zhang, B. Shah, E. Lee, P.V. Bondarenko, et al., High-mannose glycans on the Fc region of therapeutic IgG antibodies increase serum clearance in humans, *Glycobiology* 21 (2011) 949–959, <http://dx.doi.org/10.1093/glycob/cwr027>.
- [46] L. Alessandri, D. Ouellette, A. Acquah, Increased serum clearance of oligomannose species present on a human IgG1 molecule, *MAbs* 4 (2012) 509–520.
- [47] T.A. Millward, M. Heitzmann, K. Bill, U. Längle, P. Schumacher, K. Forrer, Effect of constant and variable domain glycosylation on pharmacokinetics of therapeutic antibodies in mice, *Biologicals* 36 (2008) 41–47, <http://dx.doi.org/10.1016/j.biologicals.2007.05.003>.
- [48] M.M. Newkirk, J. Novick, M.M. Stevenson, M.J. Fournier, P. Apostolakis, Differential clearance of glycoforms of IgG in normal and autoimmune-prone mice, *Clin. Exp. Immunol.* 106 (1996) 259–264.
- [49] Y. Kanda, T. Yamada, K. Mori, A. Okazaki, M. Inoue, K. Kitajima-Miyama, et al., Comparison of biological activity among nonfucosylated therapeutic IgG1 antibodies with three different N-linked Fc oligosaccharides: the high-mannose, hybrid, and complex types, *Glycobiology* 17 (2007) 104–118, <http://dx.doi.org/10.1093/glycob/cwl057>.
- [50] F. Higel, A. Seidl, U. Demelbauer, M. Viertlboeck-Schudy, V. Koppenburg, U. Kronthaler, et al., N-glycan PK profiling using a high sensitivity nanoLCMS workflow with heavy stable isotope labeled internal standard and application to a preclinical study of an IgG1 biopharmaceutical, *Pharm. Res.* (2015), <http://dx.doi.org/10.1007/s11095-015-1724-0>.
- [51] M. Yu, D. Brown, C. Reed, S. Chung, J. Lutman, Production, characterization, and pharmacokinetic properties of antibodies with N-linked mannose-5 glycans, *MAbs* 1–13 (2012).
- [52] L. Liu, A. Stadheim, L. Hamuro, T. Pittman, W. Wang, D. Zha, et al., Pharmacokinetics of IgG1 monoclonal antibodies produced in humanized *Pichia pastoris* with specific glycoforms: a comparative study with CHO produced materials, *Biologicals* 39 (2011) 205–210, <http://dx.doi.org/10.1016/j.biologicals.2011.06.002>.
- [53] L. Huang, S. Biolsi, K.R. Bales, U. Kuchibhotla, Impact of variable domain glycosylation on antibody clearance: an LC/MS characterization, *Anal. Biochem.* 349 (2006) 197–207, <http://dx.doi.org/10.1016/j.ab.2005.11.012>.
- [54] J. Bongers, J. Devincenzis, J. Fu, P. Huang, D.H. Kirkley, K. Leister, et al., Characterization of glycosylation sites for a recombinant IgG1 monoclonal antibody and a CTLA4-Ig fusion protein by liquid chromatography-mass spectrometry peptide mapping, *J. Chromatogr. A* 1218 (2011) 8140–8149, <http://dx.doi.org/10.1016/j.chroma.2011.08.089>.
- [55] A.J.S. Jones, D.I. Papac, E.H. Chin, R. Keck, S.A. Baughman, Y.S. Lin, et al., Selective clearance of glycoforms of a complex glycoprotein pharmaceutical caused by terminal N-acetylglucosamine is similar in humans and cynomolgus monkeys, *Glycobiology* 17 (2007) 529–540, <http://dx.doi.org/10.1093/glycob/cwm017>.
- [56] R. Keck, N. Nayak, L. Lerner, S. Raju, S. Ma, T. Schreitmueller, et al., Characterization of a complex glycoprotein whose variable metabolic clearance in humans is dependent on terminal N-acetylglucosamine content, *Biologicals* 36 (2008) 49–60, <http://dx.doi.org/10.1016/j.biologicals.2007.05.004>.

- [57] H. Kogelberg, B. Tolner, S.K. Sharma, M.W. Lowdell, U. Qureshi, M. Robson, et al., Clearance mechanism of a mannosylated antibody-enzyme fusion protein used in experimental cancer therapy, *Glycobiology* 17 (2007) 36–45, <http://dx.doi.org/10.1093/glycob/cwl053>.
- [58] L. Liu, S. Gomathinayagam, L. Hamuro, T. Prueksaritanont, W. Wang, T.A. Stadheim, et al., The impact of glycosylation on the pharmacokinetics of a TNFR2:Fc fusion protein expressed in glycoengineered *Pichia pastoris*, *Pharm. Res.* (2012), <http://dx.doi.org/10.1007/s11095-012-0921-3>.
- [59] E. Ezan, F. Bitsch, Critical comparison of MS and immunoassays for the bioanalysis of therapeutic antibodies, *Bioanalysis* 1 (2009) 1375–1388, <http://dx.doi.org/10.4155/bio.09.121>.
- [60] W. Wang, J. Vlasak, Y. Li, P. Pristatsky, Y. Fang, T. Pittman, et al., Impact of methionine oxidation in human IgG1 Fc on serum half-life of monoclonal antibodies, *Mol. Immunol.* 48 (2011) 860–866, <http://dx.doi.org/10.1016/j.molimm.2010.12.009>.
- [61] H. Li, M. d'Anjou, Pharmacological significance of glycosylation in therapeutic proteins, *Curr. Opin. Biotechnol.* 20 (2009) 678–684, <http://dx.doi.org/10.1016/j.copbio.2009.10.009>.
- [62] L. Huang, J. Lu, V.J. Wroblewski, J.M. Beals, R.M. Riggan, In vivo deamidation characterization of monoclonal antibody by LC/MS/MS, *Anal. Chem.* 77 (2005) 1432–1439, <http://dx.doi.org/10.1021/ac0494174>.
- [63] K.R. Anumula, New HPLC assay for glycosyltransferases based on derivatization with anthranilic acid and fluorescence detection, *Glycobiology* 22 (2012) 912–917, <http://dx.doi.org/10.1093/glycob/cws067>.
- [64] M. Melmer, T. Stangler, M. Schiefermeier, W. Brunner, H. Toll, A. Rupprechter, et al., HILIC analysis of fluorescence-labeled N-glycans from recombinant biopharmaceuticals, *Anal. Bioanal. Chem.* (2010) 905–914, <http://dx.doi.org/10.1007/s00216-010-3988-x>.
- [65] M. Melmer, T. Stangler, A. Premstaller, W. Lindner, Comparison of hydrophilic-interaction, reversed-phase and porous graphitic carbon chromatography for glycan analysis, *J. Chromatogr. A* 1218 (2011) 118–123, <http://dx.doi.org/10.1016/j.chroma.2010.10.122>.
- [66] B.D. Prater, H.M. Connelly, Q. Qin, S.L. Cockrill, High-throughput immunoglobulin G N-glycan characterization using rapid resolution reverse-phase chromatography tandem mass spectrometry, *Anal. Biochem.* 385 (2009) 69–79, <http://dx.doi.org/10.1016/j.ab.2008.10.023>.
- [67] L.R. Ruhaak, C. Huhn, W.-J. Waterreus, A.R. de Boer, C. Neusüss, C.H. Hokke, et al., Hydrophilic interaction chromatography-based high-throughput sample preparation method for N-glycan analysis from total human plasma glycoproteins, *Anal. Chem.* 80 (2008) 6119–6126, <http://dx.doi.org/10.1021/ac800630x>.
- [68] K.R. Anumula, S.T. Dhume, High resolution and high sensitivity methods for oligosaccharide mapping and characterization by normal phase high performance liquid chromatography following derivatization with highly fluorescent anthranilic acid, *Glycobiology* 8 (1998) 685–694.
- [69] F. Higel, A. Seidl, U. Demelbauer, F. Sörgel, W. Frieß, Small scale affinity purification and high sensitivity reversed phase nanoLC-MS N-glycan characterization of mAbs and fusion proteins, *MAbs* 6 (2014) 894–903, <http://dx.doi.org/10.4161/mabs.29263>.
- [70] L.A. Gennaro, J. Delaney, P. Vouros, D.J. Harvey, B. Domon, Capillary electrophoresis/electrospray ion trap mass spectrometry for the analysis of negatively charged derivatized and underivatized glycans, *Rapid Commun. Mass Spectrom.* 16 (2002) 192–200, <http://dx.doi.org/10.1002/rcm.564>.
- [71] R. Haselberg, V. Brinks, A. Hawe, G.J. de Jong, G.W. Soetsen, Capillary electrophoresis-mass spectrometry using noncovalently coated capillaries for the analysis of biopharmaceuticals, *Anal. Bioanal. Chem.* 400 (2011) 295–303, <http://dx.doi.org/10.1007/s00216-011-4738-4>.
- [72] M. Nakano, D. Higo, E. Arai, T. Nakagawa, K. Takehi, N. Taniguchi, et al., Capillary electrophoresis-electrospray ionization mass spectrometry for rapid and sensitive N-glycan analysis of glycoproteins as 9-fluorenylmethyl derivatives, *Glycobiology* 19 (2009) 135–143, <http://dx.doi.org/10.1093/glycob/cwn115>.
- [73] L.A. Gennaro, O. Salas-Solano, On-line CE-LIF-MS technology for the direct characterization of N-linked glycans from therapeutic antibodies, *Anal. Chem.* 80 (2008) 3838–3845, <http://dx.doi.org/10.1021/ac800152h>.
- [74] L.A. Gennaro, D.J. Harvey, P. Vouros, Reversed-phase ion-pairing liquid chromatography/ion trap mass spectrometry for the analysis of negatively charged, derivatized glycans, *Rapid Commun. Mass Spectrom.* 17 (2003) 1528–1534, <http://dx.doi.org/10.1002/rcm.1079>.
- [75] I. Ritamo, J. Råbinä, S. Natunen, L. Valmu, Nanoscale reversed-phase liquid chromatography-mass spectrometry of permethylated N-glycans, *Anal. Bioanal. Chem.* 405 (2013) 2469–2480, <http://dx.doi.org/10.1007/s00216-012-6680-5>.
- [76] M. Wührer, C.A.M. Koeleman, A.M. Deelder, C.H. Hokke, Normal-phase nanoscale liquid chromatography-mass spectrometry of underivatized oligosaccharides at low-femtomole sensitivity, *Anal. Chem.* 76 (2004) 833–838, <http://dx.doi.org/10.1021/ac034936c>.
- [77] H. Kalay, M. Ambrosini, P.H.C. van Berkel, P.W.H.I. Parren, Y. van Kooyk, J.J. García Vallejo, Online nanoliquid chromatography-mass spectrometry and nanofluorescence detection for high-resolution quantitative N-glycan analysis, *Anal. Biochem.* 423 (2012) 153–162, <http://dx.doi.org/10.1016/j.ab.2012.01.015>.
- [78] A. Beck, E. Wagner-Rousset, M. Bussat, M. Lokteff, C. Klinguer-Hamour, J. Haeuw, et al., Trends in glycosylation, glycoanalysis and glycoengineering of therapeutic antibodies and Fc-fusion proteins, *Curr. Pharm. Biotechnol.* 9 (2008) 482–501.
- [79] C. Zupke, L.J. Brady, P.G. Slade, P. Clark, R.G. Caspary, B. Livingston, et al., Real-time product attribute control to manufacture antibodies with defined N-linked glycan levels, *Biotechnol. Prog.* 31 (2015) 1433–1441, <http://dx.doi.org/10.1002/btpr.2136>.
- [80] C.D. Rillahan, A. Antonopoulos, C.T. Lefort, R. Sonon, P. Azadi, K. Ley, et al., Global metabolic inhibitors of sialyl- and fucosyltransferases remodel the glycome, *Nat. Chem. Biol.* 8 (2012) 661–668, <http://dx.doi.org/10.1038/nchembio.999>.
- [81] K.M. Cox, J.D. Sterling, J.T. Regan, J.R. Gasdaska, K.K. Frantz, C.G. Peele, et al., Glycan optimization of a human monoclonal antibody in the aquatic plant *Lemma minor*, *Nat. Biotechnol.* 24 (2006) 1591–1597, <http://dx.doi.org/10.1038/nbt1260>.
- [82] A. Beck, O. Cochet, T. Wurch, GlycoFi's technology to control the glycosylation of recombinant therapeutic proteins, *Expert Opin. Drug Discov.* 5 (2010) 95–111, <http://dx.doi.org/10.1517/17460440903413504>.
- [83] N. Yamane-Ohnuki, S. Kinoshita, M. Inoue-Urakubo, M. Kusunoki, S. Iida, R. Nakano, et al., Establishment of FUT8 knockout Chinese hamster ovary cells: an ideal host cell line for producing completely defucosylated antibodies with enhanced antibody-dependent cellular cytotoxicity, *Biotechnol. Bioeng.* 87 (2004) 614–622, <http://dx.doi.org/10.1002/bit.20151>.
- [84] Y. Kanda, H. Imai-Nishiya, R. Kuni-Kamochi, K. Mori, M. Inoue, K. Kitajima-Miyama, et al., Establishment of a GDP-mannose 4,6-dehydratase (GMD) knockout host cell line: a new strategy for generating completely non-fucosylated recombinant therapeutics, *J. Biotechnol.* 130 (2007) 300–310, <http://dx.doi.org/10.1016/j.jbiotec.2007.04.025>.
- [85] C. Ferrara, P. Brünker, T. Suter, S. Moser, U. Püntener, P. Umaña, Modulation of therapeutic antibody effector functions by glycosylation engineering: influence of Golgi enzyme localization domain and co-expression of heterologous β 1, 4-N-acetylglucosaminyltransferase III and Golgi α -mannosidase II, *Biotechnol. Bioeng.* 93 (2006) 851–861, <http://dx.doi.org/10.1002/bit.20777>.
- [86] T. Shinkawa, K. Nakamura, N. Yamane, E. Shoji-Hosaka, Y. Kanda, M. Sakurada, et al., The absence of fucose but not the presence of galactose or bisecting N-acetylglucosamine of human IgG1 complex-type oligosaccharides shows the critical role of enhancing antibody-dependent cellular cytotoxicity, *J. Biol. Chem.* 278 (2003) 3466–3473, <http://dx.doi.org/10.1074/jbc.M210665200>.
- [87] E. Mössner, P. Brünker, S. Moser, U. Püntener, C. Schmidt, S. Herter, et al., Increasing the efficacy of CD20 antibody therapy through the engineering of a new type II anti-CD20 antibody with enhanced direct and immune effector cell-mediated B-cell cytotoxicity, *Blood* 115 (2010) 4393–4402, <http://dx.doi.org/10.1182/blood-2009-06-225979>.
- [88] C.A. Gerdes, V.G. Nicolini, S. Herter, E. Van Puijnenbroek, S. Lang, M. Roemmele, et al., GA201 (RG7160): a novel, humanized, glycoengineered anti-EGFR antibody with enhanced ADCC and superior in vivo efficacy compared with cetuximab, *Clin. Cancer Res.* 19 (2013) 1126–1138, <http://dx.doi.org/10.1158/1078-0432.CCR-12-0989>.
- [89] A. Ito, T. Ishida, H. Yano, A. Inagaki, S. Suzuki, F. Sato, et al., Defucosylated anti-CCR4 monoclonal antibody exercises potent ADCC-mediated antitumor effect in the novel tumor-bearing humanized NOD/Shi-scid, IL-2R γ null mouse model, *Cancer Immunol. Immunother.* 58 (2009) 1195–1206, <http://dx.doi.org/10.1007/s00262-008-0632-0>.