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Research paper

N-glycans of complex glycosylated biopharmaceuticals and their impact on protein clearance

Fabian Higel[a,*], Theresa Sandl[a,1], Chi-Ya Kao[a,2], Nicole Pechinger[a], Fritz Sörgel[b,c], Wolfgang Friess[d], Florian Wolschin[a,3], Andreas Seidl[a,4]

[a] Technical Development Biosimilars, Global Drug Development Novartis, Hexal AG, Keltenring 1 + 3, 82041, Oberhaching, Germany
[b] IBMP – Institute for Biomedical and Pharmaceutical Research, Nuernberg-Heroldsberg, Germany
[c] Institute of Pharmacology, Faculty of Medicine, University Duisburg-Essen, Essen, Germany
[d] Department of Pharmacy, Pharmaceutical Technology and Biopharmaceutics, Ludwig Maximilians-Universitaet Muenchen, Munich, Germany

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ABSTRACT

N-glycosylation is a common post-translational modification of biopharmaceutical products. Certain types of N-glycans have been shown to influence important properties of monoclonal antibody products including pharmacokinetics and effector functions. Complex biopharmaceuticals e.g. Fc fusion proteins may contain several N- and O-glycosylation sites. Domain specific characterization of two Fc fusion proteins showed an Fc N-glycosylation pattern comparable to IgG molecules. The receptor N-glycosylation was found to contain some larger and more complex N-glycans compared to the Fc part. Analyses of samples from non-clinical studies of the two studied fusion proteins indicate that their N-glycans impact pharmacokinetic properties. Interestingly, besides the type of N-glycan this influence on the pharmacokinetics depends also on the glycosylation site and thus the accessibility on the protein. The same type of N-glycan can influence the clearance of fusion proteins when located at the receptor part, but not if located at the Fc part. In this study, it is shown that N-glycans with terminal galactose or N-acetylglucosamine residues have a negative impact on serum half-life when located at the receptor part. Terminal sialylation of galactose residues prevents this faster clearance even when only one sialic acid is present. O-acetylation, a modification of sialic acids does not impact pharmacokinetics. Thus, type and accessibility of N-glycan moieties of fusion proteins both play an important role in pharmacokinetics. Finally, detailed site specific analysis is critical in the development of biopharmaceuticals.

1. Introduction

ICH defines a critical quality attribute (CQA) as a physical, chemical, biological or microbiological property or characteristic that should be within an appropriate limit, range or distribution to ensure the desired product quality, safety and efficacy [1]. Influence of conserved IgG Fc N-glycosylation on effector functions (i.e. ADCC and CDC) and PK is well known and there are several reviews available covering this topic [2,3]. Certain types of Fc N-glycans are therefore CQAs of monoclonal antibody products and thus may require attention in the control strategy when manufacturing the corresponding biopharmaceuticals. High mannose N-glycans for example were shown to negatively impact half-life in circulation of IgG therapeutics across different species [4–6]. Non-fucosylated N-glycans increase the affinity to FcγRIII receptors thereby directly influencing biological activity through increased ADCC [7]. Terminal galactosylation of Fc N-glycans

Abbreviations: ADCC, antibody dependent cellular cytotoxicity; ASGPR, asialoglycoprotein receptor; AUC, area under the curve; CDC, complement dependent cytotoxicity; CHO, Chinese hamster ovary; C_{max}, maximum serum concentration; CQA, critical quality attribute; Fc, fragment cristallizable; GlcNAc, N-acetylglucosamine; ICH, International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use; IdeS, immunoglobulin-degrading enzyme from streptococcus pyogenes; IgG, immunglobulin G; MALDI-TOF, matrix-assisted laser desorption ionization – time of flight; MR, mannose receptor; PK, pharmacokinetics; SEC, size exclusion chromatography; TNF-α, tumor necrosis factor - alpha

* Corresponding author at: Analytical Development Biologicals, Boehringer Ingelheim Pharma GmbH & Co. KG, Biberach an der Riss, Germany.
E-mail address: fabian.higel@boehringer-ingelheim.com (F. Higel).
[1] Current affiliation: Biologics Process Development, Global Drug Development, Novartis Pharma AG, Klybeckstrasse 141, CH-4057 Basel, Switzerland.
[2] Current affiliation: Drug Metabolism and Pharmacokinetics, Boehringer Ingelheim Pharma GmbH & Co. KG, Biberach an der Riss, Germany.
[3] Current affiliation: Formycon AG, Fraunhoferstrasse 15, 82152 Martinsried/Planegg, Germany.
[4] Current affiliation: Leukocare AG, Am Klopferspitz 19, 82150 Martinsried, Germany.

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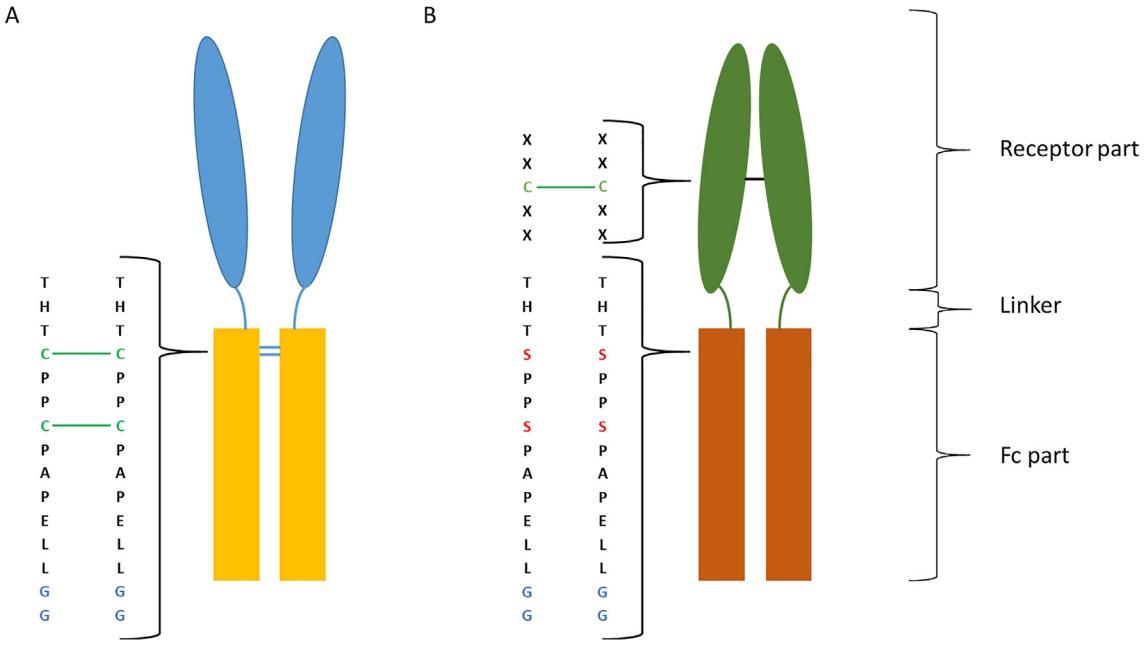

Fig. 1. Schematic drawings of fusion proteins. (A) Structure of FP1. The receptor part is fused to the Fc part (human IgG1) via a short linker sequence. Two heavy chains are connected by Fc disulfide bridges. The sequence around the disulfide bridges is shown. (B) Structure of FP2. The receptor part is fused to the Fc part (human IgG1) via a short linker sequence. The two chains are connected via a disulfide bridge between the receptor parts as illustrated. Hinge region cysteines were mutated to serine. Mutated hinge region sequence is shown.

may modulate binding to the complement protein C1q leading to a higher CDC activity [8]. Influence on PK is hypothesized to be driven by carbohydrate-specific receptors which selectively bind N-glycans of glycoproteins resulting in a faster removal of these glycovariants from circulation by endocytosis [9,10]. There are two known and well characterized lectins reported in literature that are involved in the selective uptake and clearance of glycoproteins from circulation, the asialoglycoprotein receptor (ASGPR) and the mannose receptor (MR) [9]. The ASGPR is a cell surface receptor of hepatic cells. It was demonstrated that this receptor is responsible for the specific binding to terminal galactose N-glycans and clearance of the respective glycoprotein [9,10]. Already decades ago desialylation of plasma proteins was correlated with decreased circulation half-life [11]. The MR, also referred to as N-acetylglucosamine receptor, is part of the endocytic pathway and it is also present on the cell surface of a variety of cells, including macrophages and hepatic cells [9,12]. High mannose N-glycans and N-glycans with terminal N-acetylglucosamines were reported to be bound by the MR [9,13,14]. In the case of antibodies, this binding might also depend on the pairing of N-glycans on the heavy chains. However, contradictory studies have been published on this topic [6,15].

Fc fusion proteins are closely related to mAbs. They are comprised of an IgG Fc part which can result in similar effector functions and a target binding part which is usually a receptor, receptor domain or a combination of domains of several receptors that bind with high specificity and selectivity to their ligand. In contrast to IgG molecules fusion proteins often carry several N-glycosylation sites and some also O-glycosylation sites in the non-IgG receptor part [16–19]. Influence of Fc fusion protein N-glycosylation on pharmacokinetics has been reported in a few studies [17,18,20]. Comparison of PK parameters of different batches of lenercept, an Fc fusion protein with two extracellular domains of a TNF-α receptor, showed that proteins carrying terminal N-acetylglucosamine residues were cleared faster [18]. It was hypothesized that the mannose receptor is responsible for the observed more rapid clearance as the mannose receptor had been described to bind to N-acetylglucosamine residues [18,21]. When analyzing lenercept *Jones et al.* similarly came to the conclusion that terminal GlcNAc residues may lead to a higher clearance rate [17]. A low resolving MALDI-TOF approach was utilized to compare glycopeptides site specific at selected time points. Terminal sialic acids and terminal galactosylation could not be correlated with an increased clearance [17]. In a study of an antibody-enzyme fusion protein produced in *pichia pastoris* with a high portion of high mannose N-glycans, mainly M8 and M9, it was suggested that this fusion protein was substantially cleared via the mannose receptor [20]. In a glyco-engineered version of the same expression system *p. pastoris* was used to produce fusion proteins and mAbs which were compared to the same biopharmaceuticals produced in CHO cells in transgenic mice studies. It was shown that sialic acid content of the fusion proteins correlated with the pharmacokinetics [22,23]. All published studies analyzed the N-glycans as a mixture of Fc and receptor N-glycans or with low resolving site specific methods. But there might be substantial structural variation of the N-glycosylation sites resulting in different accessibility of the glycans to receptors. It has been reported that Fc N-glycans of a certain size, e.g. bi-antennary complex type with terminal galactosylation are buried and are probably prevented from carbohydrate receptor binding [24,25]. If the same N-glycans located at the non-IgG domain are more exposed on the protein surface these N-glycans might be accessible for carbohydrate receptor binding. It is therefore of great interest to analyze the influence of Fc fusion protein N-glycans on the PK with site specificity. This is necessary to define the critical quality attributes that need to be part of a comprehensive control strategy.

In this work we analyzed the N-glycosylation pattern of two fusion proteins with distinct N-glycan patterns with specificity for the Fc and receptor part. Both Fc parts of the fusion proteins contain standard IgG1 N-glycans. However the receptor domains of the two biopharmaceuticals differ significantly in their N-glycan composition. Samples of the two fusion proteins from non-clinical studies were analyzed with highly sensitive analytical methods and the PK profiles of individual N-glycans as well as the relative N-glycan distribution over time could be obtained. We have shown previously that non-clinical animal models such as rabbits show a similar specific clearance of N-glycans as observed with human subjects and are therefore suitable models to study the influence of N-glycans on PK [6]. We show that complex bi-antennary

N-glycans with two terminal N-acetylglucosamine or galactose residues at both arms, respectively increase the clearance from circulation. However, this does not apply to N-glycans with mixed terminal N-acetylglucosamine and galactose residues. We also demonstrate that modifications of terminal sialic acids with acetyl groups do not alter the serum half-life of the biopharmaceuticals, which was so far not described in literature.

2. Results

2.1. Drug products used in this study

The structures of the fusion proteins are schematically shown in Fig. 1. The two fusion proteins FP1 and FP2 are homo dimers both consisting of an extracellular domain of cell surface receptors fused to a human IgG1 Fc part *via* a short linker sequence. Both fusion proteins are recombinant proteins expressed in CHO cell lines. FP1's receptor domain ligand is a cytokine and the FP2 receptor domain binds to a cell surface receptor of the immunoglobulin superfamily. The two FP1 chains are connected via normal IgG1 hinge region disulfide bridges (Fig. 1A) whereas FP2 has a cysteine to serine engineered hinge region and the two chains are connected via disulfide bridges between the receptor domains (Fig. 1B). Both fusion proteins have one N-glycosylation site at the Fc part and two N-glycosylation sites at the receptor domains summing up to six N-glycosylation sites per molecule. N-glycans are not involved in target binding, thus do not influence biological activity. Molecular mass of each homo dimer molecule is approximately 105 kDa, depending on the combination of attached glycoforms.

2.2. Characterization of N-glycosylation

The N-glycosylation patterns of FP1 and FP2 were characterized applying a combination of IdeS digest, size exclusion chromatography (SEC) and N-glycan mapping by reversed phase chromatography MS. The proteins were digested with IdeS to cleave the proteins between the Fc and the receptor part. The digests were subjected to SEC to separate the domains. Eluting Fc and receptor parts were fractionated, buffer exchanged and 2-AA labeled N-glycans were prepared from the domain N-glycans as described. The resulting RP-chromatograms are shown in Fig. 2. The Fc parts of FP1 (Fig. 2*A*) and FP2 (Fig. 2*B*) are comprised of standard IgG N-glycans with A2G0F and A2F being the major N-glycan for FP1 Fc and FP2 Fc respectively. N-glycosylation of the receptor parts of FP1 (Fig. 2*C*) and FP2 (Fig. 2*D*) are more complex. The receptor of FP1 (Fig. 2*C*) contains two major groups of N-glycans with terminal N-acetylneuraminic acids either with or without a core fucose with S_AA2F being the major N-glycan (19%). Major neutral N-glycans are A2F, A2G1F and A2G0F with approximately 18%, 3% and 2% respectively. The receptor of FP2 (Fig. 2*D*) carries only fucosylated complex N-glycans with a high portion of acidic N-glycans carrying terminal N-acetylneuraminic acids. In addition to neutral N-glycans, considerable levels (~4%) of acidic N-glycans with O-acetylation of the N-acetyclneuraminic acids are also present. S_AA2F (24%) is the most abundant N-glycan, A2F (20%), A2G1F (5%) and A2G0F (3%) are the most abundant neutral N-glycans and S_A2A2F-Ac (2%) the most abundant O-acetylated N-glycan on FP 2 (Fig. 2*D*). All identified N-glycans with relative abundance in the respective protein part are listed in Table S1. Pictograms and nomenclature of all identified N-glycans are provided in Fig. 3.

2.3. Glycan PK profiling of non-clinical samples of FP1

Influence of N-glycans on PK of FP1 was investigated domain specific from samples of a non-clinical rabbit study by separating Fc and receptor domains during the affinity purification as illustrated in Fig. 4.

Fig. 5 shows the resulting N-glycan composition of the receptor (Fig. 5A) and Fc part (Fig. 5B) over the circulation time after subcutaneous administration of FP1. In the elimination relevant time starting at 18 h, the N-glycan composition at the Fc part does not change over time indicating no influence of N-glycan structures on the PK. However, at the receptor part the composition of N-glycans changes over time. The terminally galactosylated N-glycan A2F decreases from initially 27% to 15% relative abundance after 72 h. Levels of A2G0F carrying two terminal GlcNAcs decreased from initially 4% to 3% after 72 h which is within the method variability. Fig. 6 illustrates exemplarily the glycan PK profiles of four N-glycans of FP1 receptor part in comparison to the PK profile of the average molecule as determined by ligand binding assay. The glycan PK profile of A2F runs below all other profiles in the elimination phase.

2.4. Glycan PK profiling of non-clinical samples of FP2

In contrast to FP1, an efficient and complete release of the Fc parts was not possible because of the engineered hinge region of FP2. The affinity purification was therefore altered to using a human IgG-Fc specific capture reagent. The resulting N-glycan composition of all FP2 N-glycans is shown in Fig. 7. Maximum concentrations were reached between 24 and 32 h after subcuteneous administration. Levels of the terminally galactosylated N-glycan A2F and A2G0F decreased from 22% to 16% after 120 h and from 16% to 13%, respectively. In contrast, the relative contribution of A2G1F rose from 25% to 32%. The level of the most abundant O-acetylated N-glycan SA2A2F-Ac was constant over time. No differences in the absorption and distribution phase were observed.

2.5. Estimation of N-glycan PK parameters and PK impact determination

To determine the impact of individual N-glycans on the overall PK, both area under the curve (AUC) and elimination were determined for each N-glycan individually. Obtained values were normalized to each fusion protein's overall elimination rate. For FP1 relative elimination was estimated between t_{max} and 72 h for the major N-glycans S_AA2F, A2F and A2G1F. For FP2 relative elimination was estimated between t_{max} and 120 h. For FP1, the relative elimination was calculated for Fc or receptor N-glycan individually for each animal and was then averaged. To investigate the impact of neutral receptor N-glycans on PK the percentage accounting for the Fc part N-glycan was subtracted from the mixed N-glycan PK data by using the N-glycan characterization data after IdeS digest and SEC separation of the reference material as described above. Based on the assumption that there is no impact on PK from FP2 Fc N-glycans as observed for FP1 and the knowledge that there are no of O-acetylated and acidic N-glycans at the Fc part it was possible to determine PK parameters for all major N-glycans of the receptor.

Comparing these estimated elimination parameters the neutral N-glycan A2F showed a 20 and 38% increased elimination rate over time when located at the receptor of FP2 and FP1, respectively. However, since only a limited number of data points was available for the estimation of the clearance the significance of this parameter is limited which is also reflected by the contradictory results for A2G0F and A2G1F of FP1 receptor Both PK profiles are not running above the average protein concentration (Fig. 6), however clearance estimation (Table 1) indicates a slower rate. Comparison of AUC was therefore used as an additional more sensitive PK parameter to assess the impact on PK. AUC differences caused by the investigated N-glycans in comparison to the average protein concentration are listed in Table 2. Again, A2F shows the highest impact with 15 and 19% negative change in AUC for FP1 and FP2. Also A2G0F has a negative impact on AUC with 5 and 9% for FP1 and FP2. Values for A2G1F are in the range of the Fc N-glycans thus this N-glycan does not significantly impact AUC. Acidic N-glycan S_AA2F shows also no impact on AUC.

Since the levels of the O-acetylated N-glycans were too low and the resulting PK profiles showed too much variability, the results were not

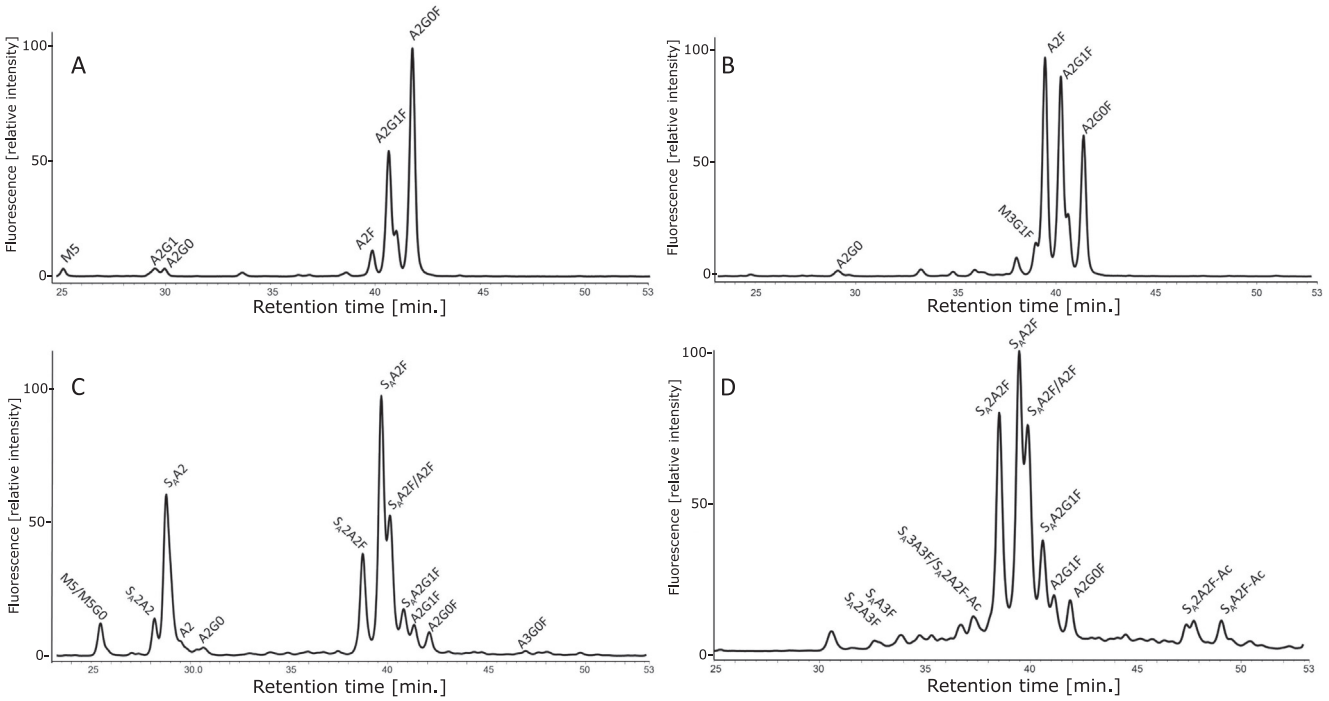

Fig. 2. Representative domain (Fc and receptor) specific glycan maps of FP1 and FP2 after IdeS digest and SEC separation. Fc glycan maps of FP 1 (A) and FP 2 (B) show a typical IgG N-glycosylation pattern comprising of predominantly complex fucosylated type N-glycans with terminal N-acetylglucosamine or galactose. Receptor glycan maps of FP1 (C) and FP2 (D) show a more complex glycosylation pattern with high portions of sialylated N-glycans. The receptor of FP1 (C) is mainly composed of acidic complex fucosylated and non-fucosylated N-glycans. The receptor of FP2 (D) are comprised solely of fucosylated complex N-glycans with a high portion of terminal N-acetylneuraminic acid. In addition FP2 receptor (D) contains N-glycans with O-acetylated sialic acids. Most abundant N-glycans are labeled. Pictograms are shown in Fig. 3. Masses and abundance of all identified N-glycans is part of the supplementary material.

suitable for sensitive determination of elimination rate and AUC. The impact on PK was assessed using the relative portion at each time point (Table 3). Mean levels vary within the standard deviation indicating no trending towards a faster or slower elimination.

3. Conclusion

Domain specific characterization data showed that the distribution of N-glycans is not homogenous on Fc fusion proteins. This was expected as the N-glycans at the Fc part are buried between the two heavy chains leaving only limited space. Therefore, predominantly smaller neutral N-glycans are present in the Fc part [3]. In contrast, the receptor part of both fusion proteins contained larger and acidic N-glycans. This circumstance is most likely due to the more exposed position of N-glycosylation sites on the protein surface. The impact on PK from different glycan variants was investigated for both FPs, for one of them site-specifically. In the current study, complex type Fc N-glycans showed no influence on PK, which is in agreement with available literature for IgG molecules [4,6,26,27]. At the receptor part of both fusion proteins a negative impact on PK was observed for two N-glycans. The strongest negative impact on PK was observed for the terminal galactosylated N-glycan A2F, which decreased the AUC of both fusion proteins by 15 and 19 percent. Terminal galactosylated N-glycans are bound by the asialoglycoprotein receptor leading to an elimination of the glycoprotein from circulation [9]. The affinity of the asialoglycoprotein receptor (ASGR) decreases with a decreasing number of terminal galactose residues [10]. The affinity for A2F, which has two terminal galactose moieties, was reported to be in the μM range and described as low [10]. Possibly, A2G1F has a too low affinity for the receptor to cause a selective clearance and therefore does not negatively influence the FP PK. The increased elimination rate of glycoproteins due to terminally galactosylated N-glycans (after desialylation) was already described in the 1970s [11].

The N-glycan A2G0F had a negative impact on AUC thus serum half-life as well, but the observed influence was not as strong as for A2F. This finding for A2G0F with two terminal N-acetylglucosamine residues is in agreement with the literature. For fusion protein lenercept an inverse correlation between A2G0F and the serum half-life of complex glycoproteins was demonstrated [17,18]. With our domain specific analysis we were able to pinpoint this influence to A2G0F located at the receptor part. The MR, a transmembrane lectin, is most likely responsible for the selective clearance of A2G0F as it contains several carbohydrate recognition domains able to bind mannose and N-acetylglucosamine residues on glycoproteins [13]. The observed parallel increase of A2G1F on FP2 cannot be explained with the obtained data. However, it is likely a consequence of the relative decrease of A2F and A2G0F if all three N-glycans are present at the same N-glycosylation site. A specific enzymatic degradation of A2F to A2G1F is unlikely, because A2G1F of FP1 did not increase whereas A2F of FP1 did decrease significantly over time and there was no degalactosylation of any N-glycan observed.

Terminal sialic acids seem to prevent binding of the ASGPR and MR since no change of sialic acid containing N-glycans over time was observed in our study. The number and branch of sialic acids attached to the N-glycan do not play a role for the studied bi-antennary N-glycans. One sialic acid attached to the N-glycan is sufficient to prevent carbohydrate receptor binding. This may be caused by the size increase of the N-glycan by the additional bulky sugar preventing the fit of the N-glycan into the receptor binding pocket or by the introduced negative charge at the N-glycan resulting in stronger repulsive forces between receptor and N-glycan.

A common yet functionally not completely understood modification of sialic acids is O-acetylation. O-acetylation converts alcohol groups of sialic acids into esters thereby reducing the polarity and hydrophilicity of the N-glycan [28]. Our data suggests that there is no direct impact of O-acetylation on the elimination. This finding has not been described in

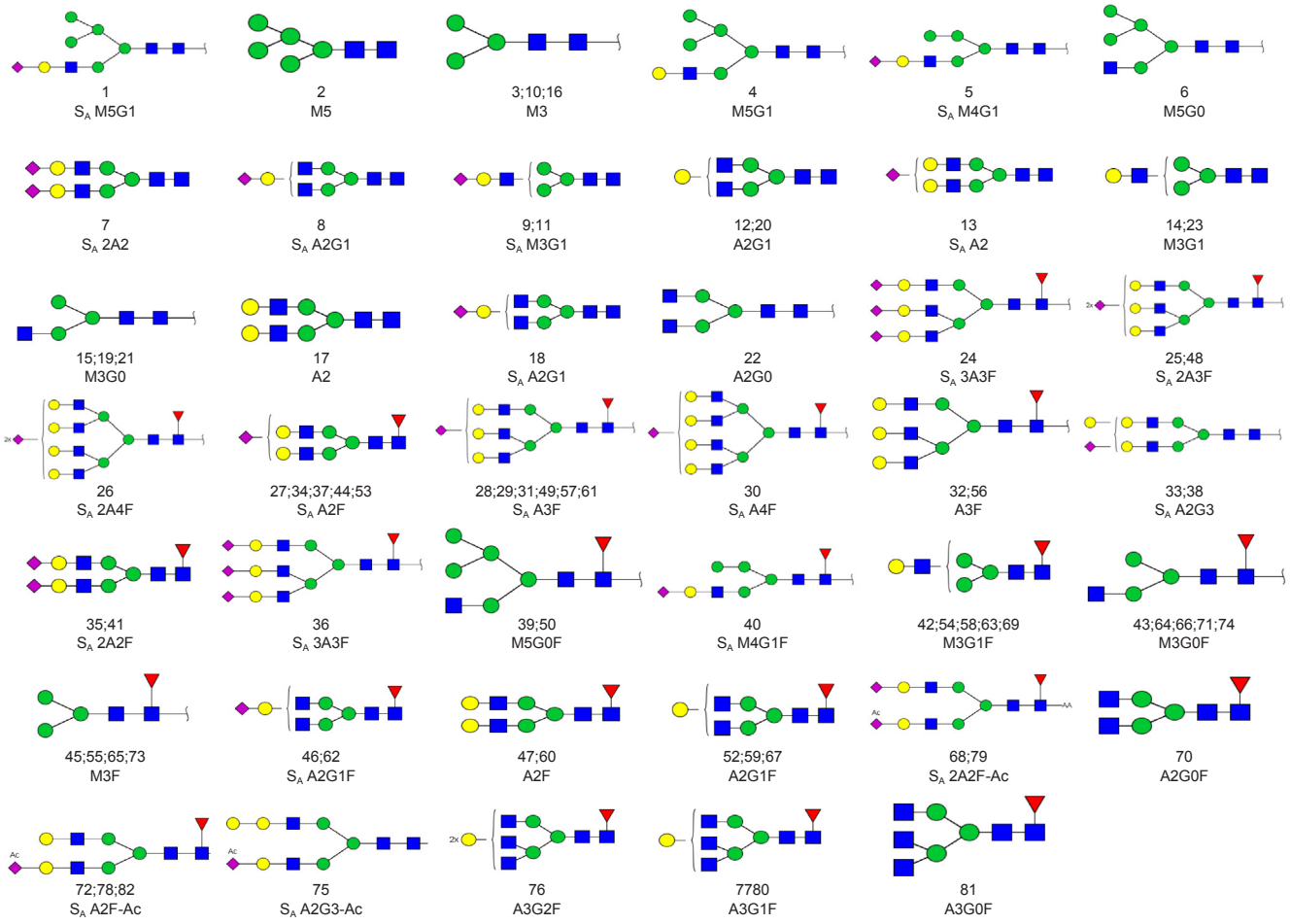

Fig. 3. Pictograms and naming of all N-glycans identified in fusion protein 1 and fusion protein 2. Only one possible name for isomers is depicted. Blue square stands for N-acetylglucosamine, green circle for mannose, yellow circle for galactose, red triangle for fucose and purple diamond for N-acteylneuraminic acid. Numbering is according to Table S1. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

literature before.

Several N-glycans of IgG molecules are considered to be critical quality attributes. This assignment is generally accepted and the impact of certain N-glycans on PK or biological activity is known. Our new data shows that for complex glycosylated biopharmaceuticals such as the investigated fusion proteins and for other non-IgG formats a comprehensive domain-specific or even site-specific characterization of N-glycosylation is required. Depending on the site and accessibility N-glycans that are uncritical for IgGs such as A2F or A2G0F can become critical quality attributes of complex glycosylated biopharmaceuticals.

4. Material and methods

4.1. Materials

PBS (Gibco; #10010023), IdeS (Genovis; #A0-FR-096), 10 kDa Amicon filters (Merck; # UFC201024), Rapid PNGaseF (New England Biolabs; P0710S), Sephadex® G-10 well plates (GE Healthcare; custom made), Picoline borane (Sigma; #654213), Anthranilic acid (Sigma; #10680), 13-C Anthranilic acid (Sigma; #709530), Acetic acid (VWR; #1.00063.1000), Acetonitrile (VWR; #1.00030.2500), DMSO (Sigma; #276855). Protein G sepharose (GE Healthcare; # 17061802), NHS activated sepharose (GE Healthcare; # 17090601), Multicreen THS HV filter plates (Merck; #MAHVN4550), 96-well polypropylene V-bottom plates (Thermo/Nunc; #249944), Capture Select™ Biotin anti-human-IgG-Fc (Thermo; #7103262100), Streptavidin magnetic beads (Thermo/Pierce; #88817)

4.2. N-glycan Characterization after IdeS digest and SEC separation

Fusion protein drug products (100 µg) were diluted in PBS to reach concentrations of 1 mg/ml. Diluted samples were incubated for 60 min at 37 °C with IdeS (100 units). Digests were subjected to size exclusion chromatography and separated under isocratic conditions. Eluting Fc/2 and receptors were collected and concentrated using spin-filters. N-glycans were released, labeled, cleaned-up and analyzed as described previously [29]. Labeled N-glycans were analyzed on a C18 BEH column (Waters) on an Agilent 1260 HPLC. Fluorescence detection was carried out with an excitation wavelength of 350 nm and emission wavelength of 440 nm. The HPLC was coupled to a Q Exactive Plus mass spectrometer (Thermo Fisher). The MS was operated with positive polarity with a spray voltage of 3.5 kV, a capillary temperature of 275 °C and an S-lens RF level of 50. Resolution was set to 70,000 with an AGC target of 1e5. Spectra were automatically processed using Expressionist software (GeneData) and N-glycans were identified according to their exact mass and retention time (in-house database). Information about N-glycan composition obtained from MS/MS data was used to support the assignment.

4.3. Preparation of ^{13}C 2-AA labeled glycan standards

^{13}C 2-AA labeled N-glycan standards were prepared from fusion protein drug product batches used in the non-clinical studies. Labeled N-glycans were prepared as described previously [29]. The fluorescence label anthranilic acid was replaced with ^{13}C-anthranilic acid.

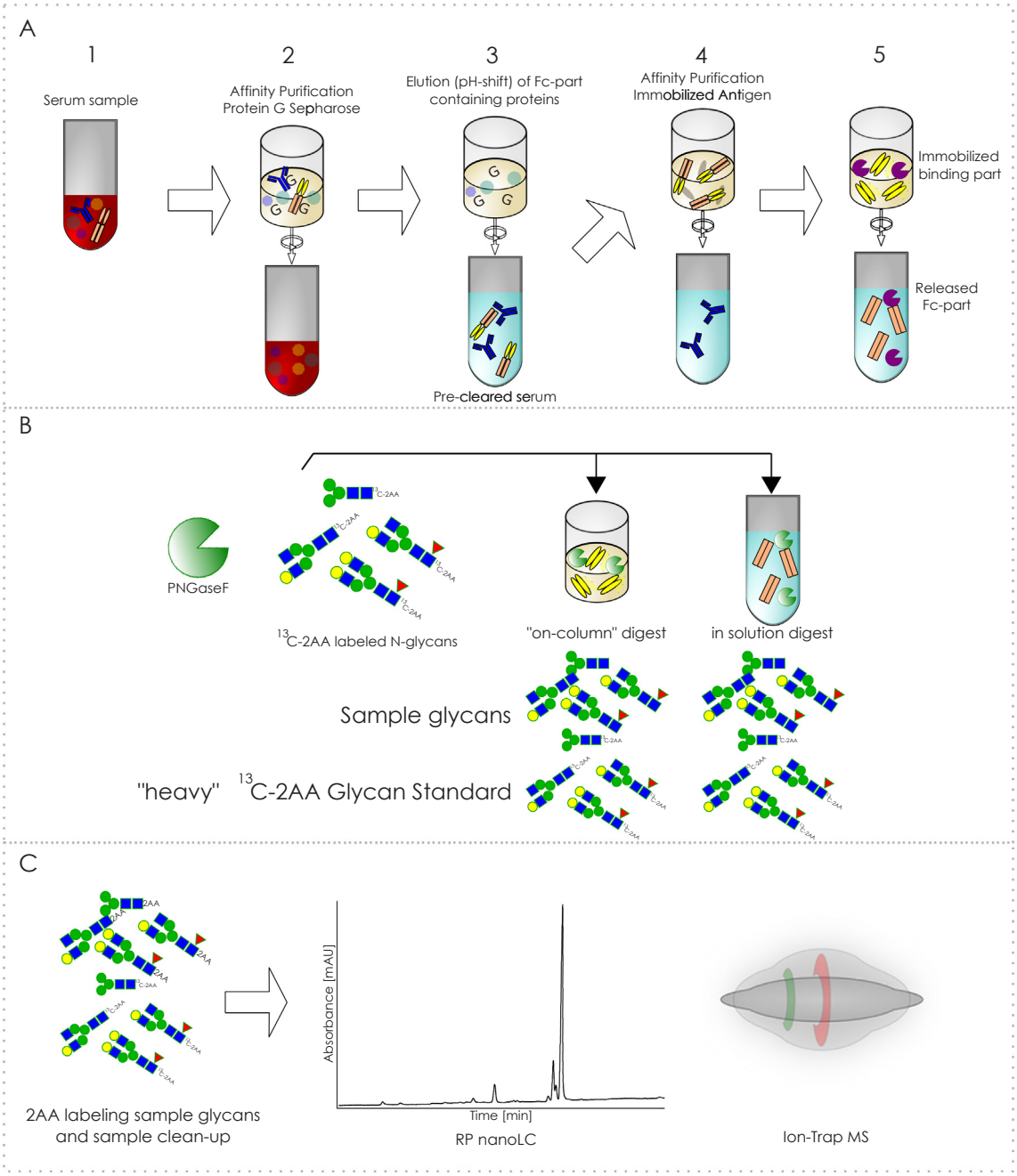

Fig. 4. Schematic work-flow of fusion protein 1 recovery from serum in a 96-well plate approach, separation of Fc and receptor part and N-glycan analysis. (A) Serum IgGs and fusion protein 1 were recovered from serum using Protein G chromatography (2). Proteins were eluted with a pH shift (3). Neutralized pre-cleared serum was then subjected to a second affinity purification with immobilized antigen (4). Fc parts were eluted with an on-column IdeS digest while receptor parts remain bound to the antigen on the affinity resin. On-column digest was achieved by centrifugation of enzyme solutions into the 96-well plate affinity columns at 30 rpm (5). (B) N-glycans were released with PNGaseF on column and in solution. With PNGaseF also a heavy isotope labeled 13C 2-AA N-glycan standard was added to each sample. (C) Released N-glycans were labeled with 2-AA as described in the method section and analyzed by nanoLC-MS.

4.4. Recovery of FP1 from non-clinical samples and N-glycan preparation

Protein G sepharose slurry (200 µL) was added to wells of a 96-well filter plate. Protein G mini columns were equilibrated with PBS. Liquid was removed by centrifugation. Affinity column plates were prepared as follows: Membranes of a 96 well filter plate were wetted with 1 mM HCl before addition of NHS activated sepharose-isopropanol slurry. Isopropanol was removed by centrifugation and the columns were washed with 1 mM HCl. Reconstituted antigen solution (1 mg/ml) was centrifuged into the columns and the coupling reaction took place for 2 h at ambient temperature. Affinity columns were washed, remaining NHS groups were inactivated with ethanolamine buffer and column plates were equilibrated with PBS. Serum samples (50 µL) were centrifuged through the equilibrated Protein G column plates. After washing the column with PBS bound IgGs and fusion proteins were eluted with a 0.1 M glycine buffer pH 2.7. Eluate was immediately neutralized with Tris HCl pH 8.0 and centrifuged through the affinity column plates. After washing the columns with PBS IdeS solution (100 units) was centrifuged into the columns to release the glycosylated Fc part of the fusion protein. Reaction was performed at 37 °C for 30 min. Released Fc parts were eluted with PBS. PNGaseF with ^{13}C-2-AA labeled N-glycan standards were added to the eluted Fc parts and to the affinity

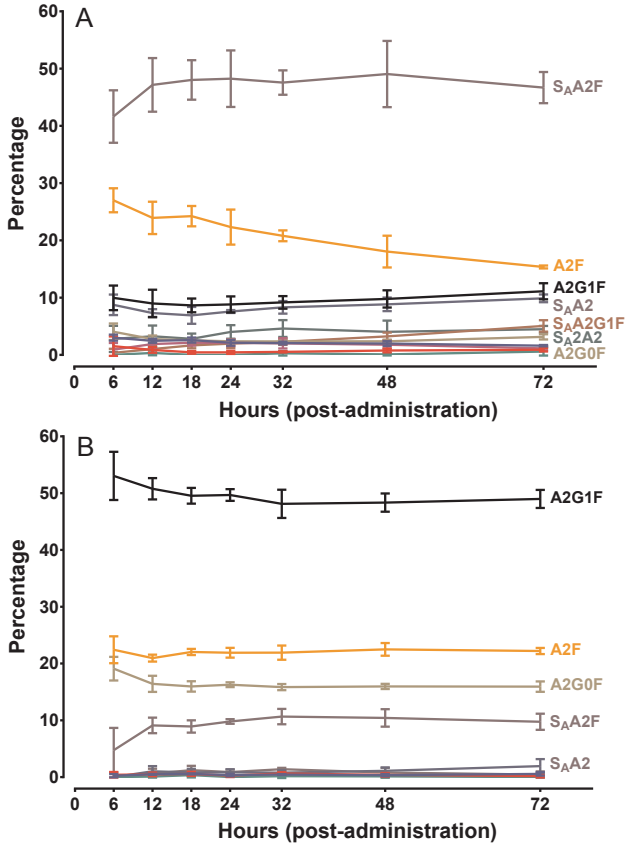

Fig. 5. Domain specific N-glycan PK data (mean and SD plotted; n = 5) of FP1 receptor (A) and Fc part (B). Levels of high abundant A2F N-glycan and low abundant A2G0F at the receptor (A) decrease over time. This decrease is compensated by increasing levels of acidic N-glycans (S_AA2F; S_AA2; S_A2G1F; S_A2A2). Levels of N-glycans at the Fc part (B) are constant over time. At 6 h the concentration of FP1 is low therefore the variability is high. Magnified views of the region below 10% are provided in Figs. S1 and S2.

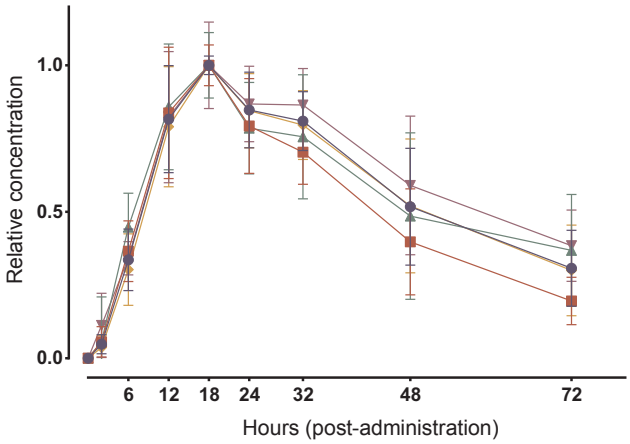

Fig. 6. Relative glycan PK profiles of A2F (red squares), A2G0F (green triangle), A2G1F (purple triangle) and S_AA2F (orange diamond) of FP1 receptor in comparison to the average molecule as determined by a ligand binding assay (blue circle). Before reaching c_{max} all PK profiles are similar. During the elimination phase the PK profile of A2F runs below the other N-glycan PK profiles. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

columns with the antigen bound receptor. Digests were incubated for 17 h at 37 °C. The N-glycans released from the antigen bound protein part were eluted with PBS from the affinity column. Proteins were removed by ultrafiltration using 96-well plates with 10 K cut-off membranes. Released N-glycans with ^{13}C glycan standards were dried by vacuum centrifugation. Dried sample N-glycans and ^{13}C 2-AA labeled glycan standard were dissolved in H2O and 2-AA labeling solution (100 mg/mL picoline borane, 50 mg/mL 2-AA in a 7:3 mixture of DMSO and acetic acid) was added. Labeling reaction took place for 17 h at 37 °C. Labeled samples were loaded to water equilibrated 96-well plate Sephadex G-10 columns and eluted with water. Samples were brought to dryness by vacuum centrifugation and dissolved in 20 µL water for nanoLC-MS analysis.

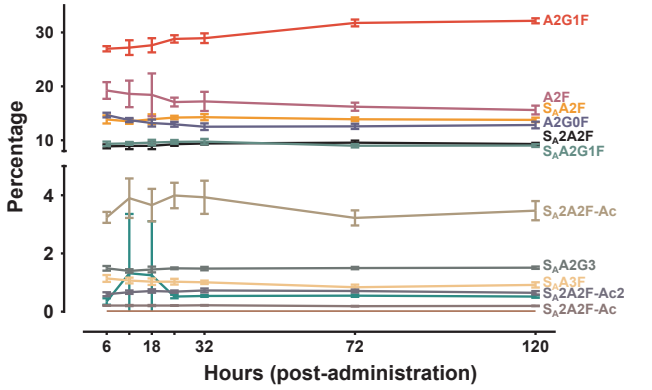

Fig. 7. N-glycan PK data (mean and SD plotted; n = 6) of FP2. Levels of N-glycans with O-acetylated sialic acids (S_A2A2F-Ac; S_A2A2F-Ac2; S_AA2F-Ac) are constant over time. Levels of A2F and A2G0F decrease over time whereas levels of A2G1F increase over time. A separation into receptor and fc part was not possible for FP2 PK data. See main text for details.

Table 1
Relative elimination rates of different N-glycans (average; number of animals: fusion protein 1 n = 5; fusion protein 2 n = 6).

N-glycan	FP1 receptor	FP1 Fc	FP2[1]
Fusion protein[2]	1.00	1.00	1.00
A2F	1.38	1.05	1.20
A2G0F	0.89	1.04	0.87
A2G1F	0.86	1.01	0.84
S_AA2F	1.03	1.04	1.03
S_A2AG2F-Ac	Not present	Not present	x[3]

[1] Receptor N-glycans only; Fc N-glycans assumed to have a relative elimination of 1.00. Ratio of Rec:Fc N-glycans based on initial distribution.
[2] Overall elimination rate.
[3] Abundance too low for sensitive analysis.

Table 2
Impact on AUC of different N-glycans compared to overall elimination of the fusion proteins (average; number of animals: fusion protein 1 n = 5; fusion protein 2 n = 6).

N-glycan	FP1 receptor	FP1 Fc	FP2[1]
A2F	−15%	< 0.5%	−19%
A2G0F	−5%	1%	−9%
A2G1F	−1%	< 0.5%	−2%
S_AA2F	+1%	x[2]	−2%
S_A2AG2F-Ac	Not present	Not present	x[2]

[1] Receptor N-glycans only; Fc N-glycans assumed to have a relative clearance of 1.00.
[2] Abundance too low for sensitive analysis.

4.5. Recovery of FP2 from non-clinical samples and N-glycan preparation

Biotin capture Select anti-human-IgG-Fc was coupled to Streptavidin magnetic beads in PBS. Beads were mixed with 40 µL

Table 3
Serum levels of O-acetylated N-glycans in FP 2. Average +/− SD (n = 6) in percent reported.

N-glycan	6 h	12 h	18 h	24 h	32 h	72 h	120 h
S_A2A2F-Ac	3.24 ± 0.19	3.90 ± 0.68	3.66 ± 0.56	3.99 ± 0.44	3.93 ± 0.57	3.22 ± 0.26	3.47 ± 0.33
S_A2A2F-Ac2	0.60 ± 0.07	0.67 ± 0.06	0.71 ± 0.06	0.69 ± 0.04	0.73 ± 0.07	0.71 ± 0.05	0.65 ± 0.06
S_AA2F-Ac	0.21 ± 0.01	0.21 ± 0.02	0.21 ± 0.02	0.21 ± 0.01	0.22 ± 0.01	0.19 ± 0.01	0.20 ± 0.01
S_AA2F-Ac2	LOD(0.01)	LOD(0.01)	LOD(0.01)	LOD(0.01)	LOD(0.01)	LOD(0.01)	LOD(0.01)
Sum	**4.06 ± 0.21**	**4.80 ± 0.73**	**4.59 ± 0.64**	**4.91 ± 0.44**	**4.89 ± 0.56**	**4.14 ± 0.27**	**4.34 ± 0.36**

LOD: limit of detection.

serum samples in 96-well plates. Binding took place at 1100 rpm and ambient temperature in a plate shaker. Plate was put onto a 96-well plate magnet and serum was removed. Beads were washed several times with PBS pH 7.4 and PBS pH 8.5. Liquid was removed from beads by placing the plate onto the 96-well plate magnet. Fusion protein-2 was deglycosylated with PNGaseF while bound to the magnetic beads. Beads were separated using a magnet and released N-glycans were mixed with ^{13}C glycan standards and dried by vacuum centrifugation. Dried sample N-glycans and ^{13}C 2-AA labeled glycan standard were dissolved in H2O and 2-AA labeling solution (100 mg/mL picoline borane, 50 mg/mL 2-AA in a 7:3 mixture of DMSO and acetic acid) was added. Labeling reaction took place for 17 h at 37 °C. Labeled samples were loaded to water equilibrated 96-well plate Sephadex G-10 columns and eluted with water. Samples were brought to dryness by vacuum centrifugation and dissolved in 20 µL water for nanoLC-MS analysis.

4.6. Glycan analysis by NanoLCMS

NanoLC (Thermo/Dionex Ultimate 3000) was set-up in "preconcentration" mode according to the manufacturer manual with a preconcentration column (3 µm particles, 75 µm × 2 cm) and an analytical column (2 µm particles, 75 µm × 25 cm). The column compartment was held at 40 °C. The mobile phase of the nano pump consisted of 0.5% formic acid in H2O (component A) and 0.5% formic acid in 50% ACN (component B). The mobile phase of the capillary pump consisted of 0.5% formic acid and 1% ACN in H2O (component C). The analytical column was equilibrated with 2% component B at a flow rate of 300 nl/min. The preconcentration column was equilibrated with 100% component C. With a user defined injection routine 8 µL sample were stacked between loading solution (0.1% formic acid, 1% ACN in ultrapure H2O) in a 20 µL sample loop. The sample loop was switched for 2 min in-line of the capillary pump flow to allow optimal trapping. Prior to the next injection sample the loop was washed with loading solution. After trapping the pre-concentration column was switched into the nano pump flow and component B was raised to 30% over 60 min, then to 95% over 5 min. After holding at 95% component B for 5 min the column was finally re-equilibrated at 2% component B for 15 min. The column outlet was connected to a UV detector with a 3 nl flow-cell. UV detection was conducted at 254 nm to monitor the preconcentration step, which is visible by an intensity drop due to the lower formic acid concentration of the loading solution. The outlet of the nanoLC was directly coupled to a Q Exactive Plus mass spectrometer (Thermo Fisher) equipped with an EASY-Spray™ Source. The source was operated in positive ionization mode with a capillary voltage of 1.5 kV, a capillary temperature of 250 °C and an S-lens RF level of 50. Resolution was set to 70,000 with an AGC target of 1e5. The MS was operated in full scan mode.

4.7. Data interpretation of nanoLCMS N-glycan PK data

Intensities of monoisotopic peaks of each labeled N-glycan and its corresponding isotopically labeled N-glycan was obtained from full scan MS data using extracted ion chromatograms. The light to heavy ratio (L/H) was determined according to Eq. (1). Where light is the sample and heavy the heavy isotope standard.

$$L/H_{(Glycan\ \ 1)} = \frac{Peak\ \ Area\ \ light_{Glycan\ \ 1}}{Peak\ \ area\ \ heavy_{Glycan\ \ 1}} \quad (1)$$

This L/H ratio was determined for each N-glycan and time point. Plotting the average L/H profile for each N-glycan provided the individual PK profiles as depicted in Fig. 6.

Glycan maps were calculated for each time point individually using the determined L/H ratio and the percentage of the respective glycan in the heavy isotope labeled standard (see Table S1) according to Eq. (2).

$$Abundance_{Glycan\ \ 1} = \frac{L/H_{Glycan\ \ 1} \times Percentage\ \ Glycan\ \ 1_{isotope\ \ standard}}{\sum_1^n (L/H_{Glycan\ \ 1} \times Percentage\ \ Glycan\ \ 1_{isotope\ \ standard} + \cdots + L/H_{Glycan\ \ n} \times Percentage\ \ Glycan\ \ n_{isotope\ \ standard})} \quad (2)$$

Competing financial interests

Chi-Ya Kao and Nicole Pechinger are employees of the Novartis group of companies which develops, manufactures and markets biopharmaceutical products. Fabian Higel and Theresa Sandl were employees of the Novartis group when this study was conducted and are currently employees of Boehringer Ingelheim Pharma GmbH which develops, manufactures and markets biopharmaceutical products. Florian Wolschin was an employee of the Novartis group when this study was conducted and is currently an employee of Formycon AG, which develops biopharmaceutical products. Andreas Seidl was an employee of the Novartis group when this study was conducted and is currently an employee of Leukocare AG.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejpb.2019.03.018.

References

[1] ICH, Pharmaceutical Development Q8(R2), ICH Harmon. Tripart. Guidel. 8 (2009) 1–28.
[2] D. Reusch, M.L. Tejada, Fc glycans of therapeutic antibodies as critical quality attributes (CQAs), Glycobiology 25 (2015) 1–45, https://doi.org/10.1093/glycob/cwv065.
[3] F. Higel, A. Seidl, F. Sörgel, W. Friess, N-glycosylation heterogeneity and the influence on structure, function and pharmacokinetics of monoclonal antibodies and Fc fusion proteins, Eur. J. Pharm. Biopharm. 100 (2016) 94–100, https://doi.org/10.1016/j.ejpb.2016.01.005.
[4] A.M. Goetze, Y.D. Liu, Z. Zhang, B. Shah, E. Lee, P.V. Bondarenko, G.C. Flynn, High-mannose glycans on the Fc region of therapeutic IgG antibodies increase serum clearance in humans, Glycobiology 21 (2011) 949–959, https://doi.org/10.1093/glycob/cwr027.
[5] L. Alessandri, D. Ouellette, A. Acquah, M. Rieser, D. LeBlond, M. Saltarelli, C. Radziejewski, T. Fujimori, I. Correia, Increased serum clearance of oligomannose species present on a human IgG1 molecule, MAbs 4 (2012) 509–520, https://doi.org/10.4161/mabs.20450.
[6] F. Higel, A. Seidl, U. Demelbauer, M. Viertlboeck-Schudy, V. Koppenburg, U. Kronthaler, F. Sörgel, W. Frieß, N-glycan PK profiling using a high sensitivity nano LCMS work-flow with heavy stable isotope labeled internal standard and application to a preclinical study of an IgG1 biopharmaceutical, Pharm. Res.

(2015), https://doi.org/10.1007/s11095-015-1724-0.
[7] R.L. Shields, J. Lai, R. Keck, L.Y. O'Connell, K. Hong, Y.G. Meng, S.H.A. Weikert, L.G. Presta, Lack of fucose on human IgG1 N-linked oligosaccharide improves binding to human Fcgamma RIII and antibody-dependent cellular toxicity, J. Biol. Chem. 277 (2002) 26733–26740, https://doi.org/10.1074/jbc.M202069200.
[8] J. Hodoniczky, Z.Z. Yuan, D.C. James, Control of recombinant monoclonal antibody effector functions by Fc N-glycan remodeling in vitro, Biotechnol. Prog. 21 (2005) 1644–1652, https://doi.org/10.1021/bp050228w.
[9] G. Ashwell, J. Harford, Carbohydrate-specific receptors of the liver, Annu. Rev. Biochem. 51 (1982) 531–554, https://doi.org/10.1146/annurev.bi.51.070182.002531.
[10] R.J. Stockert, The asialoglycoprotein receptor: relationships between structure, function, and expression, Physiol. Rev. 75 (1995) 591–609 http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2946427&tool=pmcentrez&rendertype=abstract.
[11] 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 < http://www.ncbi.nlm.nih.gov/pubmed/5545089 > .
[12] P.R. Taylor, S. Gordon, L. Martinez-Pomares, The mannose receptor: linking homeostasis and immunity through sugar recognition, Trends Immunol. 26 (2005) 104–110, https://doi.org/10.1016/j.it.2004.12.001.
[13] S.J. Lee, S. Evers, D. Roeder, A.F. Parlow, J. Risteli, L. Risteli, Y.C. Lee, T. Feizi, H. Langen, M.C. Nussenzweig, Mannose receptor-mediated regulation of serum glycoprotein homeostasis, Science 295 (2002) 1898–1901, https://doi.org/10.1126/science.1069540.
[14] J. Zhang, T. Zhang, L. Jiang, D. Hewitt, Y. Huang, Y.-H. Kao, V. Katta, Rapid identification of low level glycation sites in recombinant antibodies by isotopic labeling with (13)c(6)-reducing sugars, Anal. Chem. 84 (2012) 2313–2320, https://doi.org/10.1021/ac202995x.
[15] Y.D. Liu, G.C. Flynn, Effect of high mannose glycan pairing on IgG antibody clearance, Biologicals 44 (2016) 163–169, https://doi.org/10.1016/j.biologicals.2016.02.003.
[16] J. Bongers, J. Devincentis, J. Fu, P. Huang, D.H. Kirkley, K. Leister, P. Liu, R. Ludwig, K. Rumney, L. Tao, W. Wu, R.J. Russell, 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, https://doi.org/10.1016/j.chroma.2011.08.089.
[17] A.J.S. Jones, D.I. Papac, E.H. Chin, R. Keck, S.A. Baughman, Y.S. Lin, J. Kneer, J.E. Battersby, 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, https://doi.org/10.1093/glycob/cwm017.
[18] R. Keck, N. Nayak, L. Lerner, S. Raju, S. Ma, T. Schreitmueller, S. Chamow, K. Moorhouse, C. Kotts, A. Jones, Characterization of a complex glycoprotein whose variable metabolic clearance in humans is dependent on terminal N-acetylglucosamine content, Biologicals 36 (2008) 49–60, https://doi.org/10.1016/j.biologicals.2007.05.004.
[19] A. Beck, E. Wagner-Rousset, M. Bussat, M. Lokteff, C. Klinguer-Hamour, J. Haeuw, L. Goetsch, T. Wurch, A. Van Dorsselaer, N. Corvaïa, Trends in glycosylation, glycoanalysis and glycoengineering of therapeutic antibodies and Fc-fusion proteins, Curr. Pharm. Biotechnol. 9 (2008) 482–501 < http://www.ncbi.nlm.nih.gov/pubmed/19075687 > .
[20] H. Kogelberg, B. Tolner, S.K. Sharma, M.W. Lowdell, U. Qureshi, M. Robson, T. Hillyer, R.B. Pedley, W. Vervecken, R. Contreras, R.H.J. Begent, K.A. Chester, Clearance mechanism of a mannosylated antibody-enzyme fusion protein used in experimental cancer therapy, Glycobiology 17 (2007) 36–45, https://doi.org/10.1093/glycob/cwl053.
[21] D.T. Achord, F.E. Brot, W.S. Sly, Inhibition of the rat clearance system for agalactoorosomucoid by yeast mannans and by mannose, Biochem. Biophys. Res. Commun. 77 (1977) 409–415, https://doi.org/10.1016/S0006-291X(77)80213-1.
[22] L. Liu, A. Stadheim, L. Hamuro, T. Pittman, W. Wang, D. Zha, J. Hochman, T. Prueksaritanont, 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, https://doi.org/10.1016/j.biologicals.2011.06.002.
[23] L. Liu, S. Gomathinayagam, L. Hamuro, T. Prueksaritanont, W. Wang, T.A. Stadheim, S.R. Hamilton, The impact of glycosylation on the pharmacokinetics of a TNFR2: Fc fusion protein expressed in glycoengineered pichia pastoris, Pharm. Res. 30 (2013) 803–812, https://doi.org/10.1007/s11095-012-0921-3.
[24] 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, https://doi.org/10.1016/S0022-2836(02)01250-0.
[25] D. Houde, Y. Peng, S.A. Berkowitz, J.R. Engen, Post-translational modifications differentially affect IgG1 conformation and receptor binding, Mol. Cell. Proteom. 9 (2010) 1716–1728, https://doi.org/10.1074/mcp.M900540-MCP200.
[26] L. Alessandri, D. Ouellette, A. Acquah, Increased serum clearance of oligomannose species present on a human IgG1 molecule, MAbs. 4 (2012) 1–12 < http://www.es.landesbioscience.com/journals/mabs/article/20450/?show_full_text=true > .
[27] 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, https://doi.org/10.1093/glycob/cwn120.
[28] A. Klein, P. Roussel, O-Acetylation of sialic acids, Biochimie 80 (1998) 49–57, https://doi.org/10.1016/S0300-9084(98)80056-4.
[29] 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, https://doi.org/10.1007/s00216-012-6690-3.