Mol Biotechnol (2013) 53:326–335
DOI 10.1007/s12033-012-9531-x

RESEARCH

Generation and Comparative Characterization of Glycosylated and Aglycosylated Human IgG1 Antibodies

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Published online: 17 March 2012

Abstract Monoclonal antibodies (mAbs) are the fastest growing class of biopharmaceuticals reflecting their diverse applications in research and the clinic. The correct glycosylation of mAbs is required to elicit effector functions such as complement-dependent and antibody-dependent cell-mediated cytotoxicity, although these may be undesirable for the treatment of certain chronic diseases. To gain insight into the properties of glycan-deficient mAbs, we generated and characterized six different aglycosylated human IgG1 mAbs (carrying the N297A mutation) and compared them to their glycosylated counterparts. We found no differences in solubility or heterogeneity, and all mAbs the remained stable in stress tests at 4 and 37 °C. Surface plasmon resonance spectroscopy showed no differences in binding affinity, and the in vivo terminal serum half-life and plasma clearance were similar in rats. However, differential scanning calorimetry revealed that the aglycosylated mAbs contained a less stable C_H2 domain and they were also significantly more susceptible to pH-induced aggregation. We conclude that aglycosylated mAbs are functionally equivalent to their glycosylated counterparts and could be particularly suitable for certain therapeutic applications, such as the treatment of chronic diseases.

Keywords Glycosylation · Human IgG1 · Comparative characterization · Monoclonal antibody

Abbreviations

T_m	Transition midpoint
ADCC	Antibody-dependent cell-mediated cytotoxicity
CDC	Complement-dependent cytotoxicity

Introduction

Antibodies are complex proteins that have a conserved overall structure but a highly variable antigen-binding region. The incredible diversity of the variable region confers individual and specific binding activities that make mAbs useful as research tools, diagnostic reagents, and efficacious targeted drugs, whereas the common structure means that mAbs can be produced using standardized platform processes. This makes them beneficial to patients and profitable for biopharmaceutical manufacturers [1, 2]. Reflecting these unique merits, approximately 30% of all drugs in the development pipeline are thought to be antibodies or antibody-based molecules [3–5].

Antibody-based drugs for use in humans are usually based on isotype IgG1, a 150-kDa protein comprising two identical heavy chains and two identical light chains [6]. The Fab (antigen binding) region is responsible for divalent antigen binding, whereas the Fc (constant) region confers intrinsic biological properties. The Fc region includes an N-glycosylation site within the C_H2 domain, and the glycan structure is thought to influence the solubility, stability, immunogenicity, propensity to aggregate, Fcγ receptor

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binding, and terminal half-life in vivo of the antibody [7–13].

The requirement for correct glycosylation means that therapeutic mAbs must be produced in mammalian cells (e.g., CHO, HEK293 and NS0), which carry out authentic post-translational modifications. However, the glycan structures synthesized in mammalian cells differ according to species and cell type, and mammalian cells generally suffer from other drawbacks such as the potential for contamination with human pathogens, the requirement for expensive media, and limited scalability [14, 15].

Under some circumstances, effector functions are either unnecessary or undesirable, and it may be beneficial to produce antibodies as aglycosylated derivatives. Although potentially allowing more scope to use alternative platforms such as microbes and plants, these often have their own idiosyncratic glycan structures [16, 17]. Therefore, it is useful to express genetically engineered aglycosylated antibodies (in which the glycan acceptor sites have been abolished), and these can be produced using any platform, allowing the production of safer and more cost-effective therapeutic mAbs [18–22].

Although the glycosylated and aglycosylated forms of individual antibodies have been compared, there has to our knowledge been no comprehensive analysis of the glycosylated and aglycosylated forms of multiple antibodies raised against the same epitope to demonstrate general principles. We therefore characterized the glycosylated and aglycosylated forms of six mAbs raised against the same antigen and recognizing the same epitope, and report comparative data for solubility, stability, homogeneity, affinity, and pharmacokinetics. Although we found that the aglycosylated antibodies were more susceptible to pH-induced aggregation and the C_H2 domain was more thermolabile, the antigen-binding affinity and the terminal half-life in vivo were unaffected. The impact of our results on the development and production of antibody-based therapeutics is discussed.

Materials and Methods

Cloning, Expression, and Purification of Aglycosylated mAbs

HEK293-6E cells were maintained in F17 medium (Invitrogen) supplemented with 4 mM GlutaMAX (Invitrogen), 0.1 % Pluronic F-68 (Sigma), and 25 μg/ml G418 (Invitrogen). Genes encoding the antibody heavy and light chains were subcloned separately in expression vector pTT5 and co-transfected into HEK293-6E cells [23]. After 5–8 days of transient expression, the cleared supernatant was used to affinity purify the antibody on an Äkta System (Amersham Pharmacia Biotech) using a 10-ml HiTrap MabSelect Sure protein A column (GE Healthcare). Antibodies were eluted in two steps with 50 mM sodium acetate and 500 mM NaCl at pH 3.5 and pH 3.0. Combined elution fractions were neutralized using appropriate volumes of 2.5 M Tris base (pH >11). Aggregation products were removed by preparative size exclusion chromatography (SEC) on an Äkta Purifier System (GE Healthcare) using a custom-made SuperdexTM 200 50/600 column (GE Healthcare), with a mobile phase of PBS (pH 7.4) at a flow rate of 6.0 ml/min.

Quantification of IgGs from Cell Culture Supernatants

Antibody expression levels were determined by Protein A chromatography on an Agilent 1200 HPLC system. Samples were clarified by centrifugation for 5 min at 6,700×*g*. 900 μl of supernatant were mixed with 100 μl of 4.75 M NaCl and applied to a protein A column (POROS A/20 2.1 mm D x 30 mm L; Applied Biosystems) previously equilibrated with 50 mM Na_2HPO_4/NaH_2PO_4 + 150 mM NaCl (pH 7.2) at a flow rate of 1.5 ml/min. After washing with 10 column volumes of the same buffer IgGs were eluted with 50 mM Na_2HPO_4/NaH_2PO_4 + 150 mM NaCl (pH 3.1). The resulting peak was detected at OD_{280nm}, integrated and quantified using a IgG reference curve.

Comparison of mAbs Treated with PNGase F by CGE

Glycosylated and aglycosylated mAbs (5 μg) were incubated with 50 U of PNGase F (New England Biolabs) at 37 °C overnight under reducing conditions to confirm, at the protein level, that the aglycosylated antibodies lacked N-linked glycans. The reduced aglycosylated antibodies were separated by high resolution capillary gel electrophoresis (SDS-CGE) using the microfluidic LabChip GX II system (Caliper Life Sciences). Chip and sample preparation were carried out according to the manufacturer's guidelines and LabChip GX software was used to evaluate the results.

SDS-PAGE

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out to visualize proteins after stability analysis of glycosylated and aglycosylated antibodies following storage at 4 or 37 °C. Protein samples were diluted in PBS and 4× non-reducing (NuPage, Invitrogen) or reducing (Roth-Rotiload) SDS gel loading buffer and—in case of the latter one—heated at 95 °C for 5 min. Proteins were separated using precast 4–12 % gradient NuPAGE® Bis–Tris Gels (Invitrogen). Protein bands were visualized by staining with Simply Blue Safe Stain (Invitrogen).

Analytical Size Exclusion Chromatography (SEC)

Analytical SEC was performed on an Äkta System (GE Healthcare) system using a Tricorn Superdex^{TM} 200 300/10 GL column (GE Healthcare), with a mobile phase of PBS (pH 7.4) at a flow rate of 0.8 ml/min.

Cation Exchange Chromatography (CEX)

Analytical CEX was performed on an Agilent 2000 HPLC system using a Dionex ProPac WCX-10 CEX column. Samples were loaded in 20 mM MES (pH 6.0) and charge variants of the antibodies were separated with a continuous gradient of 32 column volumes length up to 20 mM MES (pH 6.0) + 500 mM NaCl. Elution was monitored at $OD_{280\ nm}$.

Differential Scanning Calorimetry (DSC)

Differential scanning calorimetry was used to compare the thermal stability of 200–600 µg of the glycosylated and aglycosylated mAbs on a VP-DSC Capillary Cell Micro Calorimeter (GE Healthcare) and the results were analyzed using Origin 7 software.

Surface Plasmon Resonance (SPR) Spectroscopy

The kinetics of antigen binding were compared by SPR spectroscopy using a Biacore T100 SPR biosensor (GE Healthcare). An anti-IgG antibody was immobilized onto the surface of a CM5 sensor chip (GE Healthcare), and various concentrations (1.56–200 nM) of each mAb prepared by serial dilution in HBS-EP buffer were injected into the flow cell. The running buffer was the same as the protein diluent buffer. Data were zero adjusted after subtraction of the reference cell signal using BIAevaluation version 2.0.3 software. All kinetic parameters, i.e., the dissociation rate constant (k_d), the association rate constant (k_a), and equilibrium dissociation constant (K_D), were determined by nonlinear regression analysis according to a 1:1 binding model using the BIA evaluation version 2.0.3 software.

Pharmacokinetic Analysis

The pharmacokinetic properties of one glycosylated and aglycosylated mAb pair were evaluated in vivo in male Wistar rats (Harlan, $n = 3$). Rats received a single intravascular (i.v.) bolus injection of 10 mg/kg mAb in a volume of 5 ml/kg into the tail vein. Blood samples were taken at various times from the right jugular vein via implanted catheters over a period of 10 days and placed into heparinized vials. Plasma was obtained by centrifugation of blood samples and stored at −80 °C until analysis by anti-human IgG ELISA.

IgG ELISA

To determine the IgG concentration in rat plasma samples we used a generic anti-human IgG ELISA. Nunc Maxisorb microtiter plates were coated with 1 µg/ml goat anti-human IgG (Fc) antibody (Jackson Immunolabs) in 50 mM sodium carbonate buffer (pH 9.5) incubated overnight at 4 °C, washed three times with PBST [PBS, pH 7.4 (Gibco), 0.05 % (v/v) Tween 20 (Daco)] and blocked with 2 % (w/v) BSA (Sigma) in PBS at room temperature for 1 h. Standards were prepared as twofold serial dilutions of the glycosylated or aglycosylated mAb, respectively, in rat plasma. Before measurement, standards, controls, and unknown plasma samples were diluted 1:50 with PBS containing 0.5 % (w/v) BSA. If necessary, unknown plasma samples were further diluted using PBS containing 2 % (v/v) rat plasma and 0.5 % (w/v) BSA. Samples were loaded in triplicates and incubated on the ELISA plates at room temperature for 1 h. After washing with PBST (3×), horseradish peroxidase (HRP)-labeled donkey anti-human IgG (H + L) (Jackson Immunolabs) was added in a 1:10,000 dilution in PBS and incubated at room temperature for 1 h followed by washing with PBST (3×). Bound human IgG was visualized using 100 µl per well of Ultra TMB (Thermo Fisher Scientific) as substrate. The color reaction was stopped by adding 50 µl 2 M H_2SO_4 per well. Finally, absorbance was read at 450 nm on a SpectraMax M2 microplate reader (Molecular Devices). Standard curve fitting and data evaluation were performed using SoftMaxPro 5.4 (Molecular Devices). The lower quantification limit of the assay in 2 % (v/v) rat plasma was 1.6 ng/ml.

PK Parameters

Pharmacokinetic parameters were determined by non-compartmental analysis (NCA) applying the log-linear trapezoidal rule for calculation of the area under the plasma concentration versus time curve (AUC) and the area under the first statistical moment curve (AUMC) using KinEx V3.1 (Bayer internal evaluation tool) [24–26]. Dose normalization was performed by dividing the AUC by the given dose in mg/kg resulting in the parameter AUC_{norm}.

Results and Discussion

Antibody Cloning, Expression, and Purification

Six glycosylated mAbs were converted into aglycosylated derivatives by replacing the Asn-297 residue with Ala

(N297A mutation), thereby eliminating the putative Asn-X-Ser/Thr N-glycosylation site within the Fc region. Otelixizumab (Tolerx) moved into Phase III clinical trials also bears the N297A mutation and this mutation was therefore chosen from the commonly used mutations N297A, N297D or N297Q [27, 28].

None of the antibodies contained additional Asn-X-Ser/Thr motifs, and mass spectrometry analysis also confirmed the absence of O-glycosylation (data not shown). The substitution was verified by sequencing (data not shown).

The antibodies were transiently expressed in HEK293-6E cells, and up to 918 mg/l of the glycosylated antibodies and up to 511 mg/l of the aglycosylated derivatives was recovered from the culture supernatant (Table 1). During purification, the glycosylated mAbs eluted preferentially at pH 3.5, whereas the aglycosylated derivatives required a pH of 3.0 for efficient elution indicating that the aglycosylated C_H2 domain interacted more strongly with the column resin (Fig. 1). Uniquely, the C_H2 domains are not involved in inter-chain protein–protein interactions and their stability is mediated exclusively by glycans [29, 30]. It therefore seems likely that the loss of the glycan chains alters the three-dimensional structure of the C_H2 domains and generates higher-affinity protein A interaction sites [31].

Table 1 Expression levels (mg/l) of glycosylated and aglycosylated mAbs

mAb	#1	#2	#3	#4	#5	#6
Glycosylated	557	602	707	918	721	547
Aglycosylated	453	437	511	230	268	501

Expression levels were determined by protein A HPLC analysis of cell culture supernatants

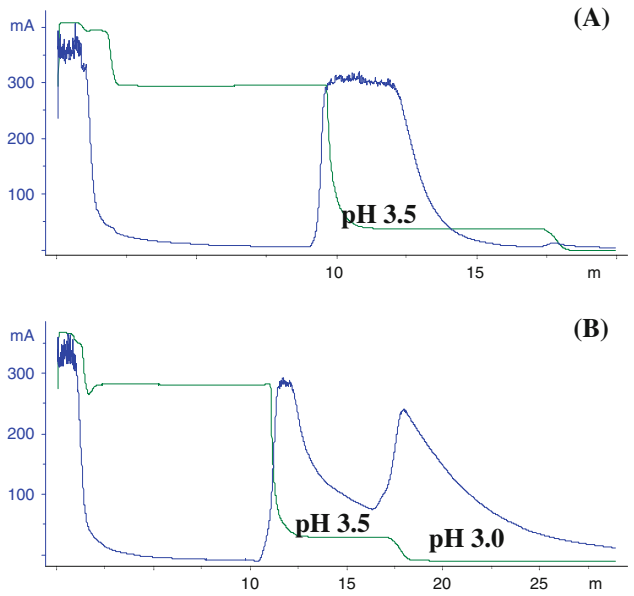

Fig. 1 Affinity purification of mAbs expressed in HEK293-6E cells (representative results are shown). **a** Elution of glycosylated mAb #1. **b** Elution of aglycosylated mAb #1. In contrast to glycosylated mAbs eluted at pH 3.5, aglycosylated mAbs required pH 3.0 for an efficient elution. *Blue line* A_{280}; *green line* pH (Color figure online)

Antibody Homogeneity, Purity and Solubility

To confirm the absence of heavy chain glycosylation at the protein level, aglycosylated and glycosylated mAbs were treated with PNGase F and compared by SDS-CGE. As anticipated, the electrophoretic mobility of the aglycosylated heavy chains was unchanged by enzyme treatment, whereas the mobility of the glycosylated heavy chains was increased by the treatment, reflecting the removal of the sugar residues (Fig. 2).

We then used CEX chromatography to investigate the distribution of post-translational micro-variants generated by processes such as the C-terminal clipping of Lys or the deamidation of Asn. All 12 mAbs produced a comparable elution profile comprising a major peak (#1) and a minor peak (#2) representing a small population of alternatively processed variants (Fig. 3; Table 2). Notably, all six aglycosylated mAbs eluted slightly later than their glycosylated counterparts indicating that the aglycosylated mAbs bound with greater affinity to the negatively charged resin, perhaps reflecting minor changes in the net surface charge.

The solubility of the glycosylated and aglycosylated mAbs and their tendency to aggregate were compared by analytical SEC after the mAbs were prepared at a final concentration of >30 mg/ml by ultrafiltration. We found that 80.7 % of the glycosylated mAbs and 76.2 % of the aglycosylated mAbs could be recovered after concentration, indicating that aglycosylation had no significant impact on solubility (Table 3). We also observed no significant formation of aggregates (data not shown).

Antibody Stability

The stability of antibodies can be affected by physical and chemical processes such as fragmentation, oxidation,

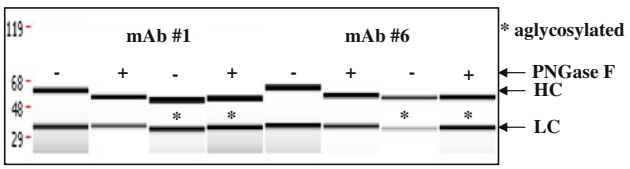

Fig. 2 PNGase F treatment of glycosylated and aglycosylated mAbs (*). SDS-CGE under reducing conditions after PNGase F treatment (+). Controls without enzyme (−). Protein markers are shown in kDa

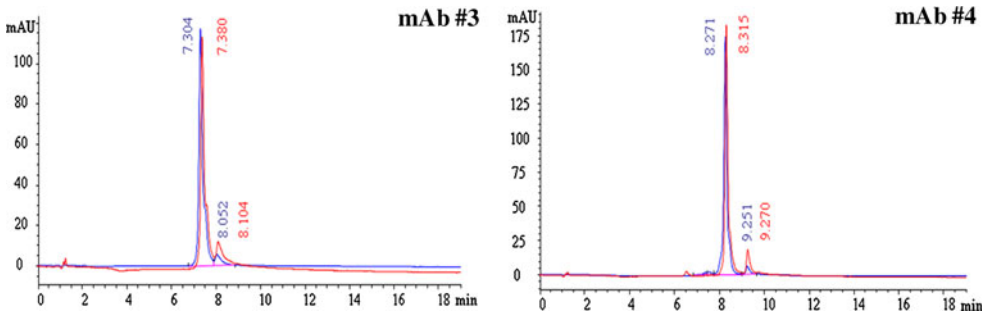

Fig. 3 Analytical CEX chromatography of glycosylated and aglycosylated mAbs (representative results are shown). *Blue line* glycosylated; *red line* aglycosylated (Color figure online)

Table 2 Quantification of different charge-variants by peak integration

Glycosylated	1st peak (%)	2nd peak (%)	Aglycosylated	1st peak (%)	2nd peak (%)
mAb #1	87.7	12.3	mAb #1	92.9	7.1
mAb #2	94.6	5.4	mAb #2	94.0	6.0
mAb #3	92.5	7.5	mAb #3	85.9	16.1
mAb #4	95.8	4.2	mAb #4	89.5	10.5
mAb #5	100	0.0	mAb #5	84.9	15.1
mAb #6	93.3	6.7	mAb #6	88.1	11.9
Average	94.0	6.0	Average	88.9	11.1

Table 3 Protein recovery after concentration

Glycosylated	Recovery (%	Aglycosylated	Recovery (%)
mAb #1	74	mAb #1	81
mAb #2	100	mAb #2	72
mAb #3	67	mAb #3	65
mAb #4	80	mAb #4	90
mAb #5	85	mAb #5	78
mAb #6	78	mAb #6	71
Average	80.7	Average	76.2

deamination, isomerization, polymerization, aggregation, and denaturation [32–38], and it is known that heat-induced antibody aggregates elicit immune responses in mice [13]. Stability during storage and administration is therefore one of the major quality attributes considered during the manufacturing and formulation of therapeutic mAbs, and this requires a comprehensive understanding of destabilization mechanisms [8, 39].

We used DSC to investigate the thermal stability of our aglycosylated and glycosylated mAbs. IgG1 molecules display a typical denaturation profile consisting of a main peak produced by the Fab fragment and a second peak derived from the C_H3 domain [40]. Crystal structures show that the C_H2 domains of glycosylated mAbs interact to form a homodimer, whereas recombinant aglycosylated C_H2 domains instead elute as a monomer when analyzed by HPLC-SEC [41]. In line with these data, we found that the C_H2 domains of our aglycosylated mAbs were prone to destabilization at temperatures as low as 62 °C compared to a T_m of ~74 °C for the corresponding glycosylated mAbs. Consequently, the denaturation profile of the aglycosylated mAbs contained an additional peak corresponding to the denatured C_H2 domains, indicating that the aglycosylated C_H2 domains are more thermolabile than their glycosylated counterparts (Fig. 4).

Low-pH treatment is a common virus-inactivation step in antibody manufacturing that typically follows affinity capture, but it is also a significant stress factor for recombinant antibodies [42]. We carried out a pH stress test to determine the stability and aggregation tendency of the glycosylated and aglycosylated antibodies under low-pH conditions, and observed that aglycosylated mAbs were significantly more susceptible to aggregation at pH 3.0 than their glycosylated counterparts (Fig. 5; Table 4). Previous studies have linked the increased and accelerated aggregation of aglycosylated mAbs to the instability of the C_H2 domain [8]. Accelerated aggregation can be induced by heat [9, 43], high concentrations of salt and low pH [8], reflecting an increase in ion-protein interactions [44–46]. The ions may be derived from the acid used for virus inactivation or from the standard salt buffer (typically 0.3–0.5 M NaCl) so aggregation could be diminished by reducing the ionic strength of the buffer as much as possible. Alternatively, aglycosylated mAbs could be expressed in microbial systems that do not support the repli cation of mammalian viruses, rendering an acid hold step unnecessary.

The thermostability of the glycosylated and aglycosylated antibodies was compared under storage conditions (21 days at 37 °C and 28 days at 4 °C, with constant agitation). All 12 mAbs remained stable under both conditions, and there were no apparent differences in fragmentation or aggregation as confirmed by SDS-PAGE and analytical SEC suggesting that the presence or absence of the glycan chains had no effect on stability at either temperature (Fig. 6).

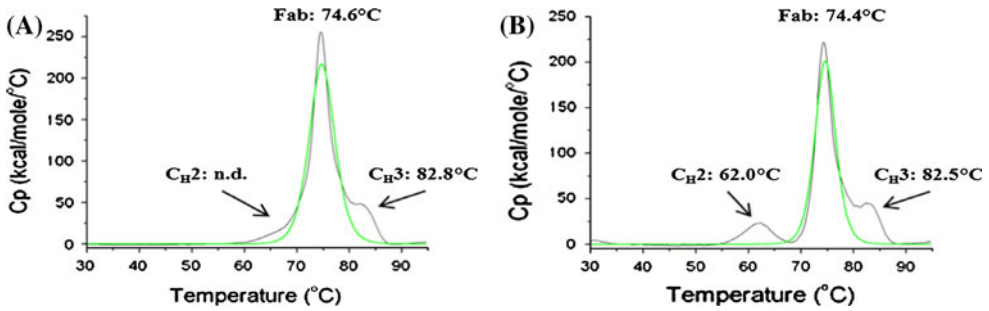

Fig. 4 Determination of thermal stability by DSC (representative results are shown). **a** Glycosylated mAb #1. **b** Aglycosylated mAb #1 with an additional C_H2 peak. T_m could be detected and quantified because of the left-shift of aglycosylated C_H2 domains. *Grey line* shows measured data; *green line* shows the fitted melting curve for the Fab fragment (Color figure online)

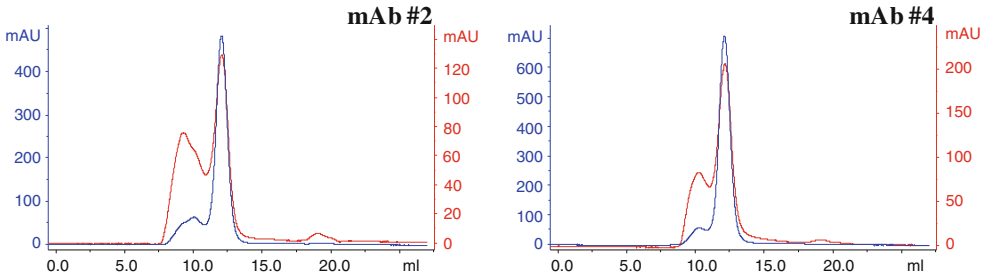

Fig. 5 Analytical SEC after a low-pH stress test (representative results are shown). *Solid blue line* A_{280} of glycosylated mAbs; *dotted red line*: A_{280} of aglycosylated mAbs. Aglycosylated mAbs showed significantly greater pH-induced aggregation (Color figure online)

Table 4 Quantitation of aggregation by peak integration

Glycosylated	Monomer (%)	Aggregate (%)	Aglycosylated	Monomer (%)	Aggregate (%)
mAb #1	100	0	mAb #1	77.4	22.6
mAb #2	82.6	17.4	mAb #2	49.4	50.6
mAb #3	94.8	5.2	mAb #3	81.4	18.6
mAb #4	91.6	8.4	mAb #4	68.5	31.5
mAb #5	90.1	9.9	mAb #5	82.9	17.1
mAb #6	86.5	13.5	mAb #6	60.2	39.8
Average	90.9	9.1	Average	70.0	30.0

Antigen Binding Analysis

Our DSC analysis revealed that the loss of glycosylation within the C_H2 domain induced conformational changes that reduced the thermal stability of the antibody (Fig. 4a, b). Because correct folding is also required for high-affinity antigen binding, we carried out SPR spectroscopy to determine the K_D values of the glycosylated and aglycosylated antibodies. We found that both variants generated similar K_D values in the range of 2.7–49 nM which indicated that the loss of glycosylation did not significantly affect antigen binding affinity (Table 5).

Pharmacokinetic Analysis

Following intravascular injection, normal human IgG antibodies equilibrate to 40 % in the vascular compartment and 60 % in the extravascular compartment, resulting in a rapid drop in the serum concentration (the α-phase). The subsequent β-phase is characterized by a slower antibody turnover, with the most stable IgG1 antibodies demonstrating a serum half-life of 20–25 days [47]. The clearance of IgG antibodies during the β-phase is controlled by various mechanisms, including the mannose and asialoglycoprotein receptors that promote clearance [48, 49], and the neonatal Fc receptor (FcRn) which protect antibodies from endosomal degradation [50, 51].

Although IgG antibodies contain only a single glycosylation site in the C_H2 domain, the glycan profile can be highly diverse both in native antibodies and in those produced as recombinant proteins in mammalian cell lines such as CHO, NS0, and SP2/0 [11]. Investigations concerning the influence of glycosylation on antibody pharmacokinetics have therefore produced greatly conflicting results. For example, whereas Newkirk et al. [52] showed that IgG1 molecules with complex type oligosaccharides

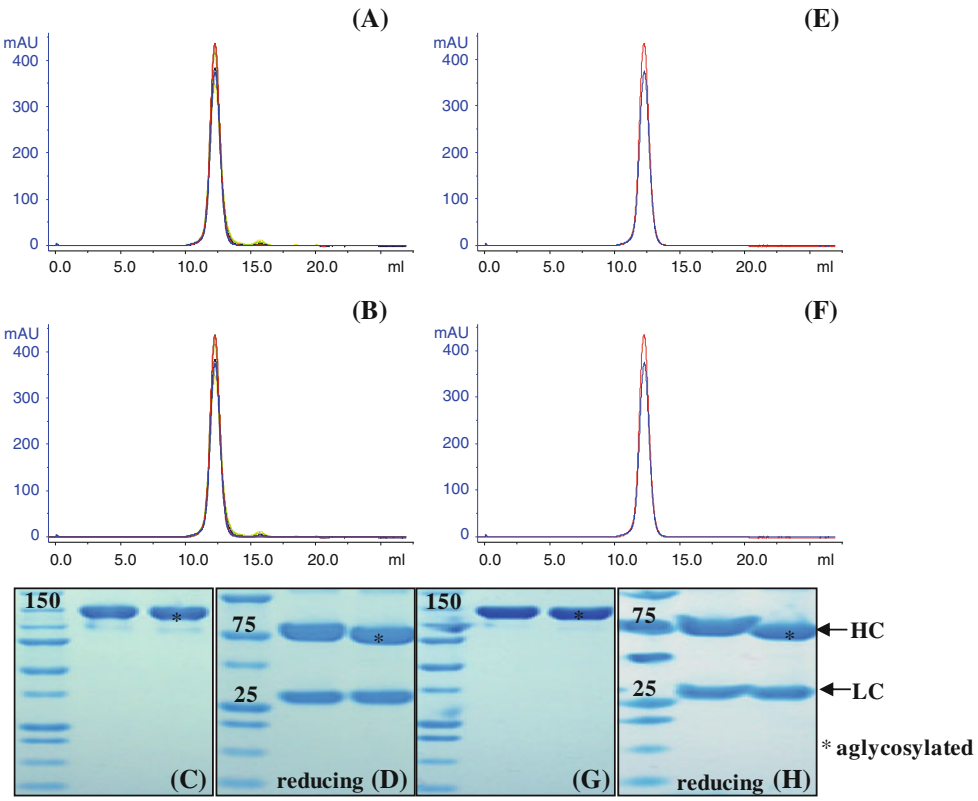

Fig. 6 Glycosylated and aglycosylated mAbs after storage at 37 °C (**a**–**d**) and 4 °C (**e**–**h**). Representative results are shown. **a** Glycosylated mAb #1 and **b** aglycosylated mAb #1 analyzed by SEC. *Blue line*: day 0; *red line*: day 1; *brown*: day 3; *black*: day 7; *green*: day 14; *yellow*: day 21. **c** Glycosylated mAb #1 and **d** aglycosylated mAb #1 analyzed by SDS-PAGE. **e** Glycosylated mAb #1 and **f** aglycosylated mAb #1 analyzed by SEC. *Blue line* day 14; *red line* day 28. **g** Glycosylated mAb #1 and aglycosylated mAb #1 analyzed by SDS-PAGE. Protein markers are shown in kDa (Color figure online)

Table 5 Surface plasmon resonance analysis of glycosylated and aglycosylated mAbs

	Glycosylated			Aglycosylated		
	k_a (1/Ms)	k_d (1/s)	K_D (nM)	k_a (1/Ms)	k_d (1/s)	K_D (nM)
mAb #1	3.10E + 05	1.52E−02	49	2.44E + 05	1.19E−02	49
mAb #2	1.70E + 05	3.43E−03	20	1.49E + 05	3.08E−03	20
mAb #3	2.07E + 05	6.73E−04	3.3	1.98E + 05	6.97E−04	3.5
mAb #4	1.90E + 05	8.20E−04	4.3	1.99E + 05	7.97E−04	4
mAb #5	1.63E + 05	8.15E−04	5	1.58E + 05	7.99E−04	5.1
mAb #6	2.08E + 05	5.65E−04	2.7	1.95E + 05	5.58E−04	2.9

had the longest serum half-life, Wright and Morrison [53] found no differences in serum half-life between antibodies with distinct glycan patterns. The balance of evidence suggests that neither Fc nor Fv glycosylation plays a critical role in determining the half-life of IgG antibodies [11].

Preliminary pharmacokinetic studies of human and humanized mAbs are routinely performed in rodents. The critical residues in the Fc region of human antibodies that bind to mouse and rat FcRn have been identified, but there are no firm data concerning potential differences in cross-species interactions [54]. We investigated the terminal half-life of a selected glycosylated/aglycosylated mAb pair (#6), ignoring glycan-dependent clearance mechanisms as these would not be relevant in the case of the aglycosylated mAbs. We found that the in vivo pharmacokinetic parameters of the glycosylated and aglycosylated antibodies were almost identical (Fig. 7). The half-life determined in the terminal phase between 48 and 240 h was 62 h for the glycosylated antibody and 64 h for its aglycosylated counterpart. The plasma clearance rate was 1 ml/h/kg for both mAbs (Table 6). In contrast to the negative effects of aglycosylation on the Fc-mediated effector functions of IgG1 antibodies, it appeared that interactions with FcRn did not depend on the presence or absence of glycans. Although it remains to be shown whether the demonstrated plasma stability is applicable to humans, we have nevertheless shown that glycosylated and aglycosylated mAbs have the same PK characteristics in rats and that glycan removal does not have a significant impact on their half-life. More detailed pharmacokinetic data could be generated by testing the stability

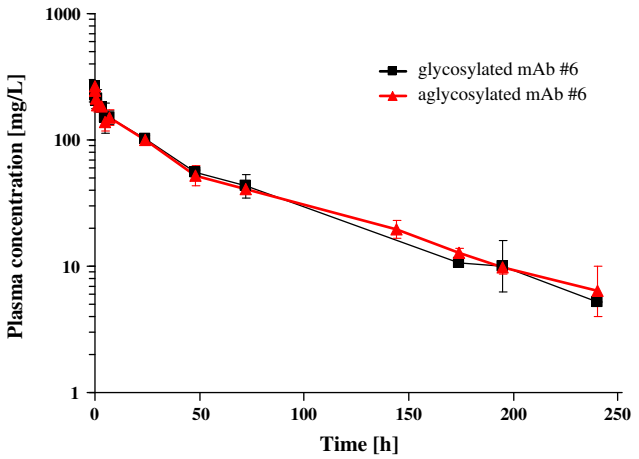

Fig. 7 Plasma concentrations of glycosylated and aglycosylated variants of mAb #6 after i.v. bolus administration of 10 mg/kg to male Wistar rats ($n = 3$). Concentrations were measured by ELISA. Terminal half-life was determined in the interval of 48–240 h

Table 6 Mean pharmacokinetic parameters in rats ($n = 3$)

mAb #6	Glycosylated	Aglycosylated
Species/sex	Rat/male	Rat/male
Strain	Wistar	Wistar
Formulation	PBS 100 %	PBS 100 %
Volume (ml/kg)	5	5
Administration route	i.v. bolus	i.v. bolus
Dose (mg/kg)	10	10
AUC (mg/h/l)	9,894	10,006
AUC_{norm} (kg/h/l)	989	1,001
CL (ml/h/kg)	1.0	1.0
V_{ss} (ml/kg)	78	78
$t_{1/2}$ (h)	62	64

IgG concentrations were determined using anti-human IgG ELISA

AUC area under the curve; AUC_{norm} AUC divided by dose; *CL* clearance; V_{ss} apparent volume of distribution at steady state; $t_{1/2}$ terminal half-life

of aglycosylated mAbs in rodents expressing the human FcRn.

Conclusions

We generated six aglycosylated human IgG1 antibodies by PCR mutagenesis and compared their physical, chemical, and biological properties to those of their glycosylated counterparts. There were only minor differences between the aglycosylated and glycosylated pairs. Both forms were soluble at concentrations up to 30 mg/ml, there was no difference in terms of structural heterogeneity and all antibodies were stable in stress tests at 37 and 4 °C as confirmed by SDS- PAGE, CGE, and analytical SEC. The aglycosylated antibodies bound more strongly to the resin during protein A affinity chromatography (requiring a more acidic elution buffer) and during CEX chromatography, suggesting a change in conformation in the protein A binding domain and a slight change in surface charge. The aglycosylated antibodies were more thermolabile when subjected to DSC analysis and were more susceptible to pH-induced aggregation. However, there were no significant differences in important functional and pharmacokinetic properties such as antigen binding affinity, terminal serum half-life, and plasma clearance rate.

Our data therefore demonstrate that aglycosylated IgG1 antibodies are functionally comparable with their glycosylated counterparts and would be valuable as drugs indicated for chronic conditions, where the presence of effector functions and the associated inflammatory response would be undesirable. Aglycosylated antibodies would therefore be useful for indications such as rheumatoid arthritis and Alzheimer's disease. The absence of glycans means that aglycosylated mAbs could be produced using microbial platforms such as Escherichia coli, which does not carry out post-translational modifications but is more economical and scalable than mammalian cells and also does not harbor human pathogens.

Acknowledgments We thank all members of Cell and Protein Sciences, Bayer Pharma AG who contributed to this manuscript. We thank Tim Brandenburger and Doris Winkler for excellent technical support in the pharmacokinetic in vivo studies and bioanalysis of plasma samples. We are especially grateful to Christoph Freiberg for support in cloning and expression. We thank Axel Harrenga for help with Biacore analysis. We also thank Richard Twyman and Fred Aswad for critical reading of the manuscript. This study was funded by BAYER Pharma AG, Wuppertal.

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