The impact of glycosylation on monoclonal antibody conformation and stability

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Key words: monoclonal antibody, glycosylation, stability, liquid chromatography-mass spectrometry, Fourier transform infrared, fluorescence spectroscopy, size-exclusion chromatography, differential scanning calorimetry

Abbreviations: ADCC, antibody-dependent cell-mediated cytotoxicity; CDC, complement-dependent cytotoxicity; CDR, complementarity determining region; CHO, Chinese hamster ovary; DSC, differential scanning calorimetry; FTIR, fourier transform infrared; GdnHCl, guanidine HCl; IgG, immunoglobulin G; LC-MS, liquid chromatography-mass spectrometry; mAb, monoclonal antibody; RP-HPLC, reversed phase high performance liquid chromatography; SEC, size-exclusion chromatography

Antibody glycosylation is a common post-translational modification and has a critical role in antibody effector function. The use of glycoengineering to produce antibodies with specific glycoforms may be required to achieve the desired therapeutic efficacy. However, the modified molecule could have unusual behavior during development due to the alteration of its intrinsic properties and stability. In this study, we focused on the differences between glycosylated and deglycosylated antibodies, as aglycosyl antibodies are often chosen when effector function is not desired or unimportant. We selected three human IgG1 antibodies and used PNGase F to remove their oligosaccharide chains. Although there were no detected secondary or tertiary structural changes after deglycosylation, other intrinsic properties of the antibody were altered with the removal of oligosaccharide chains in the Fc region. The apparent molecular hydrodynamic radius increased after deglycosylation based on size-exclusion chromatography analysis. Deglycosylated antibodies exhibited less thermal stability for the CH2 domain and less resistance to GdnHCl induced unfolding. Susceptibility to proteolytic cleavage demonstrated that the deglycosylated version was more susceptible to papain. An accelerated stability study revealed that deglycosylated antibodies had higher aggregation rates. These changes may impact the development of aglycosyl antibody biotherapeutics.

**Introduction**

Monoclonal antibodies (mAbs), having high selectivity and specificity, constitute a large and growing portion of the biotherapeutics market. The majority of marketed mAbs belong to the IgG class. IgGs, which consist of two heavy chains and two light chains linked by a total of 16 inter- or intra-molecular disulfide bonds. The two heavy chains are linked by disulfide bonds and each heavy chain is disulfide bonded to a light chain. IgGs include antigen-binding (Fab) and crystallizable (Fc) regions: the Fab is responsible for binding to the antigen, while the Fc binds to Fcγ receptors, which regulate immune responses.2

During the development of mAbs from drug candidate to marketed product, issues with stability, such as aggregation due to physical instability, or deamidation or oxidation due to chemical instability, often arise. Substantial resources and time are required to address stability problems; thus this area is one of intense focus.3,5 One factor that may also affect the stability of mAbs is the glycosylation found in the Fc region. Glycosylation is a common post-translational modification for IgG antibodies produced by mammalian cells such as Chinese hamster ovary (CHO) cells, which are frequently used for production. IgG1 molecules contain a single N-linked glycan at Asn297 in each of the two heavy chains. During the synthesis of N-glycans, multiple sugar moieties can be added to form different glycoforms, e.g., G0, G1, G2, afucosylated complex.6,7 Glycosylation plays an important role for complement-dependent cytotoxicity (CDC) and antibody-dependent cell-mediated cytotoxicity (ADCC) functions through modulating the binding to the Fcγ receptor.8,9 Particular glycoforms may be necessary to achieve therapeutic efficacy. These glycoforms may be targeted by glycosylation engineering, but may also be affected by cell culture conditions.10-12

In this report, we focus on deglycosylated antibodies. Changes in a product’s glycosylation pattern may significantly alter its intrinsic properties and stability, thereby adding challenges for downstream process development. Thus, a good understanding of the impact of glycosylation on protein properties is useful during process development. We systematically assess the impact of glycosylation on molecular structure and stability through an investigation of three IgG1 mAbs that contain a single N-linked glycan at Asn297.
glycosylation site on Asn297 of the heavy chain. We compared the intrinsic molecular properties of glycosylated and deglycosylated forms using biophysical techniques and biochemical methods after in vitro removal of the oligosaccharide chains from the antibodies using PNGase F. An accelerated stability test was also conducted to assess the potential impact on shelf-life stability.

**Results**

**PNGase F digestion.** To generate deglycosylated antibodies for our study, PNGase F was used for the in vitro removal of N-glycans. After PNGase F digestion and chromatographic purification, reduced mass data of heavy chains were obtained from liquid chromatography-mass spectroscopy (LC-MS). Spectra were derived from multiply charged ions and deconvoluted using Analyst QS 2.0/BioAnalyst 2.0 software package. The observed molecular masses of the heavy chain glycoforms for each antibody corresponded to the predicted masses (Table 1). The masses obtained for the deglycosylated antibody heavy chains confirmed the cleavage of oligosaccharide chains.

**Secondary and tertiary structure determination.** Following PNGase F treatment, a spectroscopic assessment of the higher order structure of the antibodies was performed to verify structural integrity. Fourier transform infrared (FTIR) spectroscopy was used to evaluate the secondary structure. Figure 1 shows the overlay of second derivative spectra of Amide I bands of the two forms of mAbs 1–3. The results indicated that the secondary structural change after in vitro deglycosylation was not substantial. In addition, there were no dramatic secondary structural differences among mAb1, mAb2 and mAb3, likely due to the fact that all three antibodies share the same framework and their predominant structure is β-sheet.

Intrinsic fluorescence data were also collected to detect gross changes in tertiary structure of the antibody samples. With an excitation wavelength of 295 nm, both glycosylated and deglycosylated mAb1 have emission peaks at 336 nm (Fig. 2A); both glycosylated and deglycosylated mAb2 have emission peaks at 341 nm (Fig. 2B); and both glycosylated and deglycosylated mAb3 have emission peaks at 343 nm (Fig. 2C). The fact that there are no notable differences in the fluorescence spectra of the glycosylated and deglycosylated versions of each antibody suggests that the microenvironment around the Trp residues is not substantially perturbed after PNGase F digestion. The differences in emission peak wavelengths among mAb1, mAb2 and mAb3 are presumably due to the different Trp composition in the sequence of the complementarity determining region (CDR) in the Fab region.

**Size-exclusion chromatography analysis.** After evaluating the higher order structure, we used SEC to characterize the aggregate and fragment levels of the antibodies after deglycosylation. The expanded views of SEC profiles for the antibodies are shown in Figure 3A–C. SEC profiles for each antibody remained the same after incubation at 37°C for 24 h during the PNGase F digestion. There was no notable increase in aggregate or fragment species (Table 2). However, there was a shift of the elution time of the monomer species for the deglycosylated antibodies: mAb1, mAb2 and mAb3 showed earlier elution in SEC after deglycosylation (Fig. 3A–C). This result suggests that removal of oligosaccharides could lead to an increase in the hydrodynamic radius, with the caveat that there is no change in the interaction of the antibody with the surface of the column resin.

**Thermal stability evaluation.** Thermal stability is another intrinsic property that may be important during drug development. We used differential scanning calorimetry (DSC) to evaluate the thermal stability of both glycosylated and deglycosylated antibodies (Fig. 4A–C). Previous studies have shown that antibodies normally have three thermal transitions in DSC,14,20. In Figure 4A, the first peak with the lowest transition temperature represents the thermal transition of the CH2 domain in the Fc region of mAb1; the second peak with the largest peak height represents the thermal transition of the Fab region of mAb1; the third peak with the highest transition temperature is the contribution of the CH3 domain of the Fc region of mAb1. Sometimes the thermal transitions of the Fab region and the CH3 domain are very close so that the last two peaks in the thermogram merge into one peak, such as for mAb2 (Fig. 4B). After deglycosylation, there is no significant thermal transition change for the Fab region and the CH3 domain for three mAbs as shown in Figure 4A–C. However, the transition temperature of the CH3 domain after deglycosylation is approximately 6–8°C lower compared with the glycosylated form. Figure 4A–C shows that all three thermograms have a similar transition shift in the CH3 domain. This result suggests that the presence of the oligosaccharide chain stabilizes the CH3 domain during temperature-induced unfolding and has minimal impact for other mAb regions, which confirms previous results by Mimura et al.15 The stabilization could come from the interactions between the two oligosaccharide chains or the oligosaccharide chain and the CH3 domain.15,21

**Guanidine HCl induced denaturation.** UV spectroscopy22 was used to monitor antibody unfolding induced by guanidine HCl (GdnHCl). After collecting the absorbance spectra (Fig. 5A), the second derivative of each spectrum was calculated (Fig. 5B). In this study, the negative peak around 292 nm (indicated by the arrow in Fig. 5B) from the second derivative UV was chosen as a marker to monitor the changes in the microenvironment of Trp residues.23 GdnHCl induced unfolding of glycosylated and deglycosylated mAb1 is shown in Figure 5C. Unfolding of mAb2 is shown in Figure 5D and unfolding of mAb3 is shown

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**Table 1.** Masses of mAbs’ heavy chains under reducing conditions

|                | Theoretical mass | Experimental mass |
|----------------|------------------|------------------|
| Glycosylated mAb1 | 51227.46         | 51227.57         |
| Deglycosylated mAb1 | 49783.12        | 49783.08         |
| Glycosylated mAb2 | 50419.79         | 50419.89         |
| Deglycosylated mAb2 | 48975.46        | 48975.64         |
| Glycosylated mAb3 | 50812.05         | 50812.28         |
| Deglycosylated mAb3 | 49367.71        | 49367.75         |

Error is less than 20 ppm for the experiments.
Although the increased rate of aggregate formation with the antibodies in this study is relatively modest, other aglycosyl antibodies can have markedly increased aggregation rates (data not shown). Figure 7B shows that there is no significant fragmentation rate change after deglycosylation. Thus, the higher degradation rate of the monomeric form of the antibodies in Figure 7C is mainly due to an increase in aggregation rate after deglycosylation. This study shows that removal of oligosaccharide chains can have a detrimental affect on the shelf-life stability of antibodies.

Discussion

In this study, we observed that the monomeric forms of deglycosylated mAbs elute earlier than glycosylated mAbs in SEC. This is due to either an increase in the antibody’s hydrodynamic radius after deglycosylation or a reduction in the non-specific interaction between the deglycosylated antibody and the column resin. To exclude the latter explanation, we repeated the SEC experiment with mobile phase containing 10% isopropyl alcohol, which can reduce the hydrophobic interaction between the antibody and the resin backbone; or used a Superdex 200 column instead of the TSK G3000SW_40 column, which has a different resin backbone. Results from both the SEC mobile phase modification and column resin change (data not shown) agreed with our previous finding in Figure 3A–C, i.e., monomers of deglycosylated antibodies.

Papain digestion. Previous studies\(^2\) showed that the glycosylation pattern could affect an antibody’s resistance to papain digestion. Hence, we used papain as a probe to evaluate the susceptibility to proteolytic cleavage of antibodies after oligosaccharide removal in this report. SEC was used to separate the intact mAb from digested species. The monomer content versus digestion time is plotted in Figure 6. The data show that all three antibodies are more susceptible to papain digestion after deglycosylation, with little observed difference among the three glycosylated antibodies or the three deglycosylated antibodies.

Accelerated stability. We also evaluated the stability of both glycosylated and deglycosylated antibodies under the accelerated condition for up to three months. SEC was used as the major stability-indicating assay during this study. The percentage of aggregates, fragments and monomers from SEC chromatograms are plotted separately in Figure 7A–C. Figure 7A shows that all three antibodies exhibit higher aggregation rates after deglycosylation.

In Figure 5E. The midpoints of the structural transition during the unfolding process are summarized in Table 3. All three antibodies show a similar trend: the deglycosylated form is less resistant to GdnHCl induced unfolding (the midpoint of the transition occurs at a concentration of GdnHCl that is lower by about 0.6 M). This unfolding result is consistent with the DSC data in this report that demonstrated that the glycan in the Fc region of the antibody conferred stability to the molecule.

Although the increased rate of aggregate formation with the antibodies in this study is relatively modest, other aglycosyl antibodies can have markedly increased aggregation rates (data not shown). Figure 7B shows that there is no significant fragmentation rate change after deglycosylation. Thus, the higher degradation rate of the monomeric form of the antibodies in Figure 7C is mainly due to an increase in aggregation rate after deglycosylation. This study shows that removal of oligosaccharide chains can have a detrimental affect on the shelf-life stability of antibodies.
PNGase F treatment is removal of the CH$_2$ domain glycan, this result suggests that the CH$_2$ domain is also involved during aggregate formation. Previous work by Chennamsetty et al.\textsuperscript{26} showed that there were several aggregation-prone motifs near the glycosylation site, e.g., residues Val$^{282}$, Pro$^{291}$, Tyr$^{296}$, Val$^{308}$, Leu$^{109}$ that could undergo localized conformational changes after deglycosylation. A most recent report by Kayser et al.\textsuperscript{30} also mentioned that many hydrophobic residues masked by carbohydrate were exposed after deglycosylation. Both reports used molecular dynamics simulations during their analysis and suggested that the hydrophobicity change in the CH$_2$ domain played an important role during aggregate formation. To further explore this hypothesis, we evaluated the hydrophobicity change after deglycosylation using SYPRO Orange dye as a probe. SYPRO Orange dye, which features high selectivity and sensitivity, can interact with hydrophobic regions of proteins and yields a strong fluorescence signal that is readily measured.\textsuperscript{31,32} Figure 8A–C showed that the extrinsic fluorescence intensity was increased after deglycosylation for all three antibodies, which indicated an increase in hydrophobic regions after removal of oligosaccharides. Above all, both CDRs in the Fab region and the CH$_2$ domain in the Fc region are involved in the aggregate formation, and the less stable CH$_2$ domain after deglycosylation contributes substantially to the aggregation rate increase at elevated temperatures.

On the other hand, although both CDR sequence and deglycosylation of the CH$_2$ domain showed a significant impact on antibody aggregation (Fig. 7A), there was no substantial antibodies eluted earlier than monomers of glycosylated antibodies. Thus, the antibody monomer retention time decrease in SEC after deglycosylation is most likely due to a change in hydrodynamic radius.

Computational modeling is a potential approach to reveal the mechanistic basis of phenomena associated with antibody solution phase structure and dynamics.\textsuperscript{25-28} One such study that is presently ongoing suggests that deglycosylation permits the CH$_2$-CH$_2$ domains of the Fc region to assume conformations in which these two domains are farther apart than in corresponding glycosylated structures (personal communication, TW Patapoff and JP Brandt). This phenomenon may account for the slightly larger hydrodynamic volume of deglycosylated antibodies as detected by SEC.

We used three IgG1 mAbs that were based on a human framework containing heavy chain V$^\text{HIII}$ and light chain V$^\text{I}$ subgroup sequences but with different CDR sequences in the Fab region as model proteins in this report. A previous study showed that Fab-Fab interaction played a critical role in increasing viscosity due to self-association.\textsuperscript{29} This Fab-Fab interaction could also be important during aggregate formation, as suggested by the fact that glycosylated mAb1, mAb2 and mAb3 exhibited different aggregation levels in SEC (Table 2). In addition, all three antibodies exhibited a higher aggregation rate after removal of the glycan, (Fig. 7A). Because the only change that occurs during Table 2. Size distribution of mAbs from SEC analysis\textsuperscript{1}

|               | Aggregate (%) | Monomer (%) | Fragment (%) |
|---------------|--------------|-------------|--------------|
| Glycosylated mAb1 | 0.2          | 99.7        | 0.1          |
| Deglycosylated mAb1 | 0.3         | 99.6        | 0.1          |
| Glycosylated mAb2 | 0.5          | 99.5        | 0.0          |
| Deglycosylated mAb2 | 0.6         | 99.4        | 0.0          |
| Glycosylated mAb3 | 0.9          | 99.0        | 0.1          |
| Deglycosylated mAb3 | 0.9         | 99.0        | 0.1          |

\textsuperscript{1}Error is less than 0.1% for the SEC analysis. \textsuperscript{2}Aggregate percentage in this table only represents soluble aggregates detectable by SEC. It can also be reported as high molecular weight species (HMWS).
Other researchers also reported that removal of the glycan chain could affect the elution of antibodies from protein A and protein G chromatography, which might be caused by the localized conformational change at low pH.38

A recent study from He et al. 39 showed that a deglycosylated IgG1 molecule had significantly lower stability compared to other glycosylated IgG1 or IgG2 molecules, which is similar to results from DSC (Fig. 4A–C) and GdnHCl-induced unfolding (Fig. 5C–E) experiments in this report. A potential implication for antibody drug development from our study is that unforeseen challenges may arise in the development of aglycosyl antibodies due to a decrease in stability. In summary, data in the current study indicate that the N-linked oligosaccharide in IgG1 provides an important contribution to structural stability that may be of significance during biotherapeutic development.

Materials and Methods

Antibodies. Humanized IgG1 mAbs1, 2 and 3 were produced in CHO cells and purified using standard antibody purification...
Figure 5. MAb unfolding monitored by second derivative ultraviolet absorbance spectroscopy. (A) The original UV absorbance spectrum. (B) The calculated second derivative UV spectrum. The wavelength of the negative peak around 292 nm (indicated by the arrow) was recorded during the mAb unfolding process. (C) Squares are the wavelength of the negative peak around 292 nm for glycosylated mAb1 at various GdnHCl concentrations. Triangles represent the wavelength of the negative peak around 292 nm for deglycosylated mAb1. Black curves show the transition after sigmoidal fitting. (D) Unfolding data of glycosylated (squares) and deglycosylated (triangles) mAb2. (E) Unfolding data of glycosylated (squares) and deglycosylated (triangles) mAb3.
eluent was analyzed by UV detection at 214 nm and then subjected in-line to electrospray ionization mass spectrometry (ESI MS) using an Applied Biosystems/MDS Sciex QStar Elite mass spectrometer with the time-of-flight (TOF) detection mode.

Size-exclusion chromatography analysis. SEC was performed on Agilent 1200 HPLC system using a TOSOH TSK gel G3000SW_{2} column (7.8 x 300 mm; TOSOH Bioscience, cat. 100368-358) to quantify aggregation and fragmentation. The mobile phase contained potassium phosphate and potassium chloride at pH 6.2. The column was eluted isocratically at a flow rate of 0.5 mL/min for 30 min and the column effluent was monitored at 280 nm. All data were collected at 25°C and peaks were integrated using Chromeleon software (Dionex). The percentage of aggregate, fragment or monomer species were quantified based on the total peak areas. This SEC method was used for the characterization of aggregate and fragment levels, papain digestion and accelerated stability studies in this report.

Procedures, e.g., protein-A affinity chromatography, Q-sepharose anion exchange chromatography, sulfopropyl-sepharose cation exchange chromatography, at the Genentech Oceanside facility. High purity was achieved for all antibodies and aggregate levels were less than 1%.

PNGase F digestion. PNGase F (New England Biolabs, cat. P0705L) was used to remove the N-glycan by incubating 1 mg of each antibody with 275 units of PNGase F at 37°C for 24 hours. After digestion, sulfopropyl-sepharose cation exchange chromatography was used for purification. The eluted antibodies were formulated to 20 mM histidine acetate, 0.02% polysorbate 20 solutions at pH 5.5 with a concentration of 20 mg/mL. All glycosylated and deglycosylated antibodies in this study were formulated in this buffer.

Liquid chromatography-mass spectroscopy analysis. Antibodies were diluted to 1 mg/ml using 20% acetonitrile (J.T. Baker, cat. 9017-03) and reduced with tris (2-carboxyethyl) phosphine hydrochloride (Thermo Scientific, cat. 20491) at 60°C for 10 minutes. The reduced samples were injected onto a reversed phase high performance liquid chromatography (RP-HPLC) column for direct online mass spectroscopy analysis. RP-HPLC was performed on an Agilent 1200 HPLC system. The mobile phase was water with 0.1% formic acid (Thermo Scientific, cat. 28905) and 0.025% trifluoroacetic acid (Thermo Scientific, cat. 28904) as solvent A and acetonitrile with 0.1% formic acid and 0.025% trifluoroacetic acid as solvent B. A Varian PLRP-S polymer column with 1,000 Å pore size and 8 μm particle size (4.6 x 50 mm; Varian, cat. 1572-1502) was used for the RP-HPLC analysis. The column temperature was maintained at 75°C during analysis and the flow rate was 0.5 mL/min with gradient elution. The column...
Extrinsic fluorescence spectra of the antibodies were collected using the same spectrofluorometer at 25°C. SYPRO Orange dye (Invitrogen, cat. S6651) was chosen as the extrinsic fluorescent reagent in this study. The stock solution of SYPRO Orange dye was 5,000x working concentration in dimethyl sulfoxide. During the fluorescence measurement, all samples contained 0.2 mg/mL antibody with 1x SYPRO Orange dye in 20 mM histidine acetate buffer at pH 5.5. The fluorescence spectrum was collected from 305 to 700 nm with excitation at 495 nm. A blank spectrum containing 1x SYPRO Orange dye in the same buffer was subtracted from each sample spectrum. The excitation and emission slit widths were both 5 nm and data were collected at 0.2 nm increments with 0.2 s integration time.

**Differential scanning calorimetry measurements.** DSC thermograms were collected from 10 to 100°C on a MicroCal VP DSC. Antibodies were diluted to 2 mg/mL with 20 mM histidine acetate, 0.02% polysorbate 20 buffer at pH 5.5 before loading into the sample cell. The heating rate was set to 60°C per hour. A thermogram of the formulation buffer was also collected and subtracted from each sample thermogram. Origin 7.0 software (OriginLab Corporation) was used for blank subtraction and baseline correction.

**Second derivative UV absorbance spectroscopy.** UV spectra of the antibodies were recorded using a Hewlett-Packard 8453 UV-Visible spectrophotometer during antibody unfolding. An 80 μl sample of 0.5 mg/mL antibody with variable concentrations of GdnHCl (Thermo Scientific, cat. 24115) was loaded into a 1 cm pathlength quartz cuvette. Samples were equilibrated in GdnHCl solutions for 5 min before the UV measurement. Spectra were collected from 200 to 400 nm at room temperature and a buffer blank spectrum was subtracted. Agilent UV-Visible Chemstation software was used to calculate second derivative spectra. Data were processed using Origin 7.0 software.

**Papain digestion.** Papain is a protease that cuts in the hinge region of antibodies and is frequently employed to generate Fab and Fc fragments. In this study, antibodies were mixed with...
papain (Sigma-Aldrich, cat. P5306-25MG) at 25:1 (w/w) ratio in 20 mM Tris buffer at pH 7.0 and incubated at 37°C. Time points for SEC analysis were taken at 2, 4, 6, 8, 16 and 24 h. Papain inhibitor, antipain (MP Biomedicals, cat. 152843), was added to the antibody solution at the end of each time point to quench papain activity. The ratio between papain and antipain was kept at 40:1 (w/w). Samples were analyzed by SEC immediately.

**Accelerated stability.** All glycosylated and deglycosylated mAbs were stored in 3 cc type-I glass vials with a 1 mL fill volume. Samples were filtered through a 0.22 μm filter (Millipore, cat. SLG033RS) and filled aseptically. The vials were sealed with a rubber stopper and an aluminum cap. Four vials of each glycosylated or deglycosylated mAb were kept at -70°C as study control materials for each time point. The rest of the vials were stored in a 40°C incubator out of direct light. Data were collected at the time points of t = 0, 0.5-month, 1-month, 2-month and 3-month. A fresh vial was used for each time point.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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