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REPORT

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Quantitative analysis of glycation and its impact on antigen binding

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ABSTRACT

Glycation has been observed in antibody therapeutics manufactured by the fed-batch fermentation process. It not only increases the heterogeneity of antibodies, but also potentially affects product safety and efficacy. In this study, non-glycated and glycated fractions enriched from a monoclonal antibody (mAb1) as well as glucose-stressed mAb1 were characterized using a variety of biochemical, biophysical and biological assays to determine the effects of glycation on the structure and function of mAb1. Glycation was detected at multiple lysine residues and reduced the antigen binding activity of mAb1. Heavy chain Lys100, which is located in the complementary-determining region of mAb1, had the highest levels of glycation in both stressed and unstressed samples, and glycation of this residue was likely responsible for the loss of antigen binding based on hydrogen/deuterium exchange mass spectrometry analysis. Peptide mapping and intact liquid chromatography-mass spectrometry (LC-MS) can both be used to monitor the glycation levels. Peptide mapping provides site specific glycation results, while intact LC-MS is a quicker and simpler method to quantitate the total glycation levels and is more useful for routine testing. Capillary isoelectric focusing (cIEF) can also be used to monitor glycation because glycation induces an acidic shift in the cIEF profile. As expected, total glycation measured by intact LC-MS correlated very well with the percentage of total acidic peaks or main peak measured by cIEF. In summary, we demonstrated that glycation can affect the function of a representative IgG1 mAb. The analytical characterization, as described here, should be generally applicable for other therapeutic mAbs.

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Introduction

Glycation refers to the nonenzymatic reaction between sugars and proteins as originally described by Maillard.[1] Glycation is triggered by the exposure of proteins to reducing sugars such as glucose, fructose and galactose, which typically react with the side chains of lysine residues or the *N*-termini of proteins to form a Schiff base between the aldehyde groups of the sugars and the primary amines of the protein. Formation of the Schiff base intermediate is reversible, but this intermediate can be converted into a more stable ketoamine adduct through an Amadori rearrangement.[2–4] Glycated antibodies have been detected *in vivo*,[5–7] and glycation has been observed during antibody production.[8,9] Glycation alters the charge profile of therapeutic proteins,[10] and could potentially affect the stability[11,12] and potency of the protein.[13–18]

Monoclonal antibodies (mAbs) are commonly exposed to reducing sugars at different stages of the manufacturing process.[19,20] For example, high concentrations of glucose are usually added at the beginning of the cell culture process and supplemented intermittently as needed. Galactose may also be added to the cell culture medium to control the level of galactosylation.[21] In addition to the concentration of reducing sugars, cell culture conditions such as temperature, pH, and time affect the rate and extent of glycation.[10,22–24] Glycation can also occur during storage at higher temperatures because commonly used formulation excipients such as sucrose and trehalose can convert to glucose at high temperature.[11,12,25]

Under normal manufacturing conditions, glycation is typically detected at multiple Lys residues on the antibody surface, but the level of glycation at any specific residue is typically low.[25,26] Glycation in the constant regions has been shown to have little or no detectable impact on Fc functions.[27] However, the complementary-determining regions (CDRs) are unique to each mAb and glycation in the CDR could affect antigen binding.[16,18]

In this study, we assessed the effects of glycation on the structure and function of an IgG1 mAb (mAb1), which was produced in Chines hamster ovary (CHO) cells using a fed-batch process and formulated in the absence of reducing sugars. The non-glycated and glycated species were isolated from unstressed mAb1 drug substance (DS) by boronate affinity chromatography (BAC), which allowed characterization of the glycated protein generated during cell culture rather than glycated protein produced by incubation with high concentrations of glucose. The effects of glycation on the higher order structure, charge heterogeneity, thermostability and biological functions of mAb1 were evaluated using a variety of analytical methods. Results suggested that both intact liquid chromatography-mass spectrometry (LC-MS) and peptide mapping can be used to quantify glycation levels in mAb1, and results from

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both methods correlated very well with the relative abundance of acidic peaks or the main peak measured by capillary isoelectric focusing (cIEF). Glycation had no detectable effects on Fc effector functions based on analysis of complement-dependent cytotoxicity (CDC) and antibody-dependent cell-meditated cytotoxicity (ADCC), or binding to FcγRI, FcγRIIIa and the neonatal Fc receptor (FcRn). We also observed no effects on the higher order structure of mAb1 based on hydrogen/deuterium exchange (HDX) MS, circular dichroism (CD), analytical ultracentrifugation (AUC) and differential scanning calorimetry (DSC) analysis. However, changes in antigen binding were detected using a binding assay and by HDX MS analysis.

The results of our study indicated that glycation in the CDRs can affect antigen binding and needs to be controlled, while glycation in the Fc region is of less concern. Furthermore, our results suggest that intact LC-MS can be used as a fast and quantitative assay to measure total levels of glycation, and these results correlate very well with results from peptide mapping. Finally, cIEF is able to detect changes in glycation and can be used as an alternative assay for batch release and stability testing to ensure proper control of glycation.

Results

Enrichment of glycated mAb1

In order to assess the effect of glycation on the structure and function of mAb1, glycated mAb1 was isolated from DS by BAC.[28–31] BAC separates glycated and non-glycated proteins based on the highly specific and reversible interaction (esterification) between the tetrahedral anion formed from boronic acid at alkaline pH and the 1,2-cis-diols on glycated amino acids.[32,33] As shown in Fig. 1, the non-glycated mAb1 did not bind to the column and eluted in the flow-through, while the glycated mAb1 bound to the column and was eluted with a step gradient of sorbitol. Although BAC separated the non-glycated and glycated mAb1 very well, it could not be used as a quantitative tool due to the highly variable results obtained from different lots of columns. Therefore, it was used only as a preparative method to enrich the non-glycated and glycated mAb1. Individual fractions of glycated and non-glycated mAb1 were collected, concentrated, and exchanged into mAb1 formulation buffer for subsequent characterization.

Total glycation measured by intact LC-MS analysis

Intact LC-MS analysis can be used to measure the total glycation levels in antibodies because each glycation event increases the protein mass by 162 Da (mass of a hexose sugar), which can be readily resolved and quantitated by intact mass analysis.[10,25,30] However, mAbs typically contain *N*-linked glycans with 0, 1 or 2 terminal galactose residues, which also differ in mass by 162 Da and therefore interfere with glycation quantitation. Thus, samples were treated with endoglycosidase S (EndoS) to eliminate glycan heterogeneity prior to analysis.[34,35] As shown in Fig. 2, the deconvoluted mass spectra of the mAb1 DS (top panel) contained a major peak at 145,837 Da that corresponded to the non-glycated form of mAb1 and 2 smaller peaks at 146,000 and 146,159 Da that corresponded to the

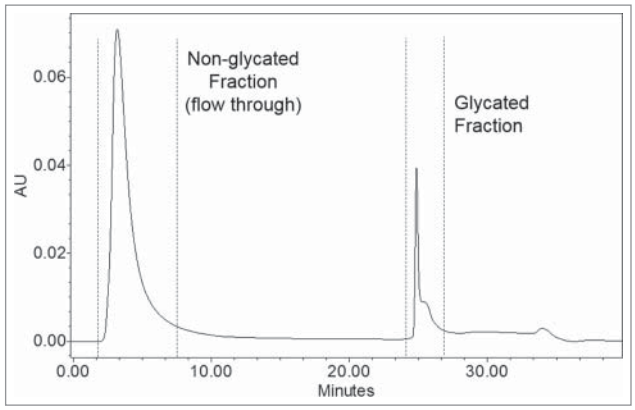


Figure 1. Separation of non-glycated and glycated mAb1 by boronate affinity chromatography. Absorbance at 280 nm is plotted as a function of time in minutes. The column fractions pooled for structure-function characterization are indicated by dashed lines.

mono- and di-glycated forms of mAb1, respectively. In contrast, the isolated non-glycated fraction (middle panel) was enriched in the non-glycated peak at 145,834 Da with a smaller, mono-glycated peak at 145,994 Da and no detectable di-glycated mAb1, whereas the isolated glycated fraction (bottom panel) was enriched in the mono-glycated peak at 145,999 Da with much smaller peaks corresponding to non-glycated, di-glycated and tri-glycated mAb1. These results indicated that most glycated mAb1 was glycated at a single site and only small amounts were glycated at 2 or 3 sites. This information cannot be obtained by peptide map analysis.

The relative abundance of each species in the three samples was calculated from peak intensities in the centroid deconvoluted mass spectra and results were expressed as a percentage of the total peak intensity. As shown in Table 1, total glycated species (the sum of mono-, di-and tri-glycated species) in the DS was 15.4%, whereas total glycated species in the non-glycated and glycated fractions was 3.1% and 89.9%, respectively. These results indicated that BAC was an effective method for enriching the glycated and non-glycated species.

Site specific glycation measured by peptide map analysis

Glycation mainly occurs on the primary amines of the *N*-terminal residues and the ε amine of Lys side chains.[36–39] Peptide mapping was performed to identify the glycated residues and quantify the glycation level of each peptide.[8,10,26,30,31] Endoproteinase Lys-C and trypsin are commonly used to digest proteins for peptide mapping, but these enzymes could not be used to quantify glycation because they do not cleave at glycated Lys residues. Thus, Asp-N was used to identify and quantify the sites of glycation in mAb1 samples. The glycated peptide peaks were initially detected by a shift in peak retention time and/or by a difference between the observed and expected peptide masses, and further confirmed by MS/MS analysis. Glycation levels for each of the peptides were quantified by peak area integration of the extracted ion chromatograms and results were expressed as a percentage of the total peak area (glycated and non-glycated peptides).

As shown in Table 2, 10 glycated peptides were detected. Some peptides contained more than 1 potential glycation

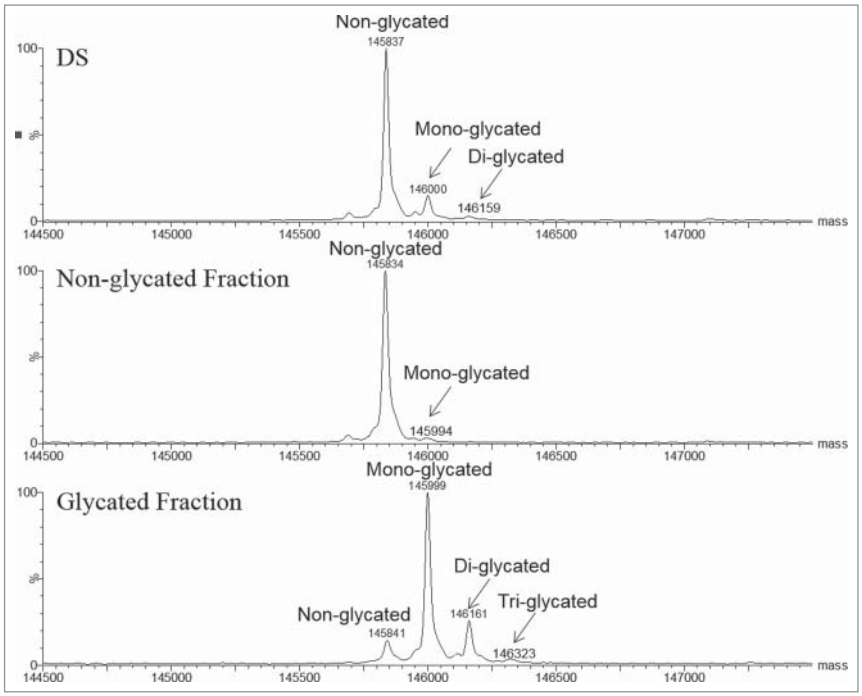


Figure 2. Intact LC-MS analysis of EndoS treated mAb1. Deconvoluted mass spectra are presented for drug substance (DS) in the top panel, the isolated non-glycated fraction in the middle panel, and the isolated glycated fraction in the bottom panel.

Table 1. Glycation levels measured by intact LC-MS for mAb1 DS, non-glycated and glycated fractions. Relative abundance (%) was presented for each species.

Species	DS	Non-Glycated Fraction	Glycated Fraction
Non-glycated	84.6	96.9	10.1
Mono-glycated	13.1	3.1	69.3
Di-glycated	2.3	ND	18.0
Tri-glycated	ND	ND	2.6
Total glycated	15.4	3.1	89.9

ND = not detected.

site, but each peptide contained only 1 modification (mono-glycated). Importantly, the levels of all glycated peptides were lower in the non-glycated fraction and higher in the glycated fraction, which again indicated that all glycated sites were successfully enriched using BAC. As shown in Fig. 3,

Table 2. Peptide map analysis of mAb1. The levels of glycated peptides are presented for the DS and the enriched non-glycated and glycated fractions.

Sites	DS	Non-Glycated Fraction	Glycated Fraction
HC Lys100	2.1	ND	21.6
HC Lys126/138/152	1.1	0.6	3.2
HC Lys 227/251/253	1.2	0.1	3.7
HC Lys293/295	0.5	0.3	5.7
HC (Lys322/325/327/331/339/343/345)	ND	ND	1.7
LC Lys39	ND	ND	1.4
LC Lys103/107	0.5	0.5	2.1
LC Lys126/145/149	0.6	0.3	2.3
LC Lys183	0.8	0.7	3.1
LC Lys188/190/207	1.1	0.4	5.0

ND = Not detected.
HC = Heavy chain.
LC = Light chain.

most of these glycated Lys residues were located on the surface of mAb1. The most sensitive site for glycation was heavy chain (HC) Lys100, which increased from 2.1% in the unfractionated DS control to 21.6% in the isolated glycated fraction. HC Lys100 is located in the antigen binding region of mAb1 (Fig. 3).

Charge heterogeneity measured by cIEF analysis

cIEF separates proteins based on differences in the isoelectric points (pIs) and is used to measure the charge heterogeneity of proteins.[40–43] Changes in glycation are expected to correlate with changes in the relative abundance of total acidic peaks or the main peak because glycation can cause a perturbation (depression) of the lysine sidechain pka, which may result in the loss of protonation depending on the pH.[10] As expected, the percentage of total acidic peaks increased from 33.8% in the mAb1 DS to 72.2% in the glycated fraction and the percentage of the main peak decreased from 61.7% in the mAb1 DS to 22.3% in the glycated fraction (Table 3 and Fig. 4). More specifically, Peak 4 increased from 7.2% in DS to 42.3% in the glycated fraction. This suggested that Peak 4 corresponded to the mono-glycated species since this was the most abundant species detected by intact LC-MS analysis of the glycated fraction (Fig. 2). There was also a new Pre-3 peak in the glycated fraction, which likely corresponded to the di-glycated species.

Correlation of glycation measurements

The results presented above indicated that intact LC-MS, peptide mapping and cIEF can all be used to measure or monitor glycation. To determine if consistent results were obtained with all three methods, mAb1 DS was incubated with increasing

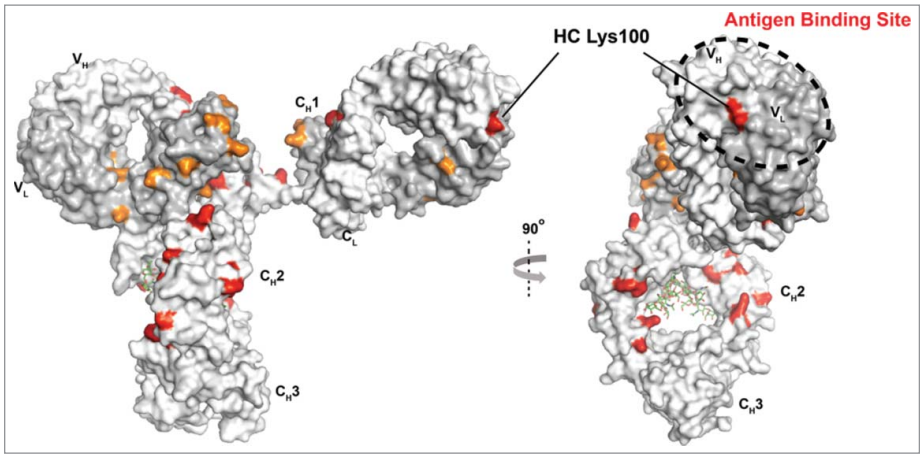


Figure 3. Location of glycated lysine residues in mAb1. The structural model is based on the crystal structure of Fab (in-house data) and homology structure (PDB: 4BYH) of Fc. Glycated heavy chain Lys residues are displayed in red; glycated light chain Lys residues are displayed in yellow. Left and right panels are two different views of the mAb1 structure with a 90° turn.

concentrations of glucose for 24 hours at 37 °C as described in the Methods section to generate samples with increasing levels of glycation. The results obtained from all three methods were then compared to determine the consistency of measurements (Fig. 5A).

As shown in Fig. 5B, there was a strong linear correlation between total glycation measured by intact LC-MS and HC Lys100 glycation measured by peptide mapping ($R^2 = 0.99$). Although peptide mapping provides quantitation of individual glycation sites, it requires a more complicated sample preparation procedure, longer LC-MS/MS runs and more extensive data analysis than intact LC-MS. It also requires analysis with a second enzyme (Asp-N) in addition to the more commonly used Lys-C or trypsin digestion for monitoring other post-translational modifications (PTMs). Therefore, intact LC-MS is a quicker and more effective method to monitor glycation. Another advantage of intact LC-MS analysis is that it measures the total level of glycation (e.g., mono-glycated, di-glycated, tri-glycated), which can be more useful for routine testing because glycation often occurs at many sites at very low levels and can be difficult to measure consistently by peptide mapping.

As shown in Fig. 5C and 5D, there were also strong linear correlations between the total acidic peaks measured by cIEF with total glycation measured by intact LC-MS ($R^2 = 0.98$) and HC Lys100 glycation measured by peptide mapping ($R^2 =$ 0.99). There were also strong, inverse linear correlations between the main peak measured by cIEF with total glycation measured by intact LC-MS ($R^2 = 0.98$) and HC Lys100 glycation measured by peptide mapping ($R^2 = 0.99$). Together, these results indicated that cIEF was a suitable assay for monitoring changes in glycation and could be used as an alternative to the two MS methods to ensure control of glycation if combined with the appropriate acceptance criteria.

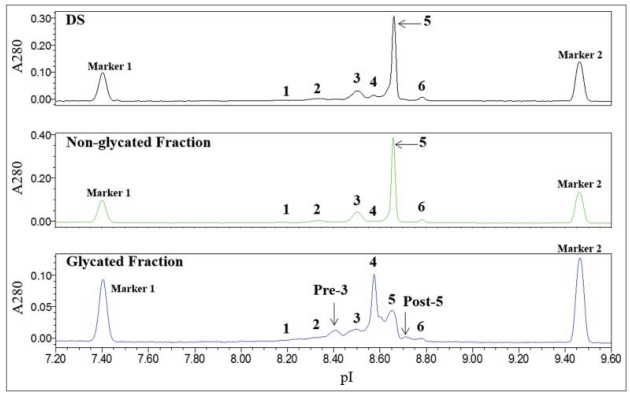


Figure 4. cIEF analysis of mAb1. cIEF electropherograms are presented for the DS, non-glycated and glycated fractions.

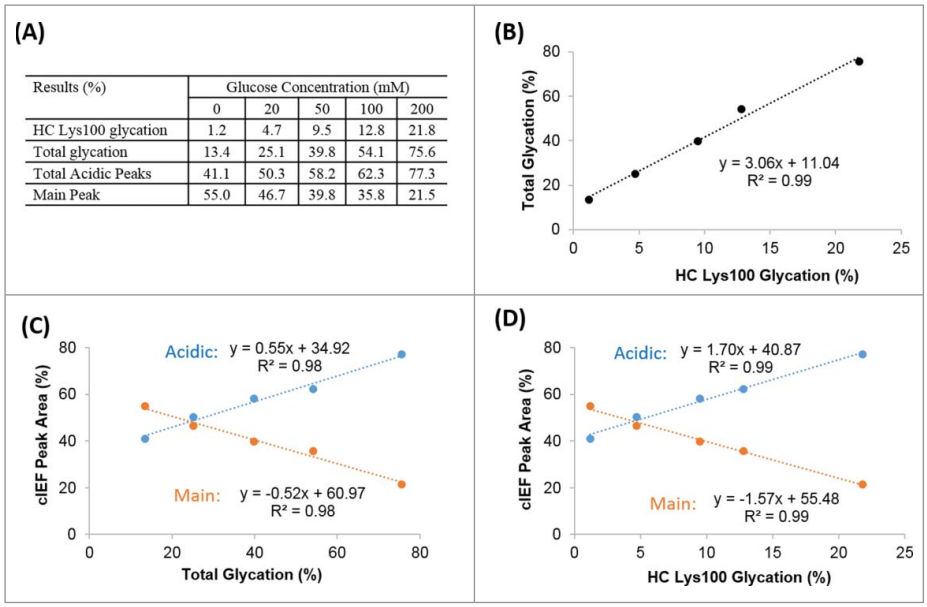
Table 3. Relative abundance (%) of cIEF peaks in mAb1 DS, non-glycated and glycated fractions.

Peak No.	DS	Non-Glycated Fraction	Glycated Fraction
1	1.0	1.6	ND
2	5.9	8.2	ND
Pre-3	ND	ND	17.7
3	19.7	20.0	12.2
4	7.2	3.5	42.3
5 (Main)	61.7	62.1	22.3
Post-5	ND	ND	3.2
6	4.6	4.6	2.4
Total Acidic Peaks	33.8	33.3	72.2
Total Basic Peaks	4.6	4.6	5.6

ND = Not detected.

Bioactivity of glycated mAb1

Antigen binding, CDC activity, ADCC activity, Fcγ receptor binding, and FcRn binding can all be critical to the mechanism of action of antibodies. The effects of glycation on these biological functions were evaluated using the isolated glycated fractions and the glucose stressed samples. As shown in Table 4, glycation had a notable impact on antigen binding, which decreased from 104% in the non-glycated fraction to 61% in the glycated fraction. A decrease in antigen binding was also observed in the glucose stressed samples. There were inverse linear corrections between antigen binding and the total glycation levels measured by intact LC-MS and HC Lys100 glycation levels measured by peptide mapping (Fig. 6). This loss of



Results (%)	Glucose Concentration (mM)				
	0	20	50	100	200
HC Lys100 glycation	1.2	4.7	9.5	12.8	21.8
Total glycation	13.4	25.1	39.8	54.1	75.6
Total Acidic Peaks	41.1	50.3	58.2	62.3	77.3
Main Peak	55.0	46.7	39.8	35.8	21.5

Figure 5. Correlation of analytical results for glycation. Results for HC Lys100 glycation measured by peptide mapping, total glycation measured by intact LC-MS, and total acidic peaks and main peak abundance measured by cIEF are shown in panel A for the glucose stressed samples. The correlation of total glycation measured by intact LC-MS with HC Lys100 glycation measured by peptide mapping is shown in panel B. The correlation of total acidic peaks and main peak measured by cIEF with total glycation measured by intact LC-MS is shown in panel C; and the correlation of total acidic peaks and main peak measured by cIEF with HC Lys100 glycation measured by peptide mapping is shown in panel D.

antigen binding was most likely due to glycation of HC Lys100, which was the most sensitive site for glycation and the only site located in a CDR of mAb1.

Small decreases in CDC activity, ADCC activity and FcγRIIIa binding were also observed in the enriched glycated fraction, but these small differences were within method variation and no differences were detected in the glucose stressed samples. Together, these results indicated that glycation had little impact on these Fc effector functions, which was consistent with results from previously studies.[27]

Impact on structure and function by HDX MS

HDX MS has been used successfully in previous studies to demonstrate the effects of PTMs on the higher order structure and receptor binding of IgG antibodies.[44–48] Thus, HDX MS was used in this study to determine if the decrease in antigen binding of glycated mAb1 was due to glycation of HC Lys100.

HDX MS was first used to compare the conformation of non-glycated and glycated mAb1. As shown in Fig. 7A, there were no significant differences in the HDX levels for any HC peptides between the non-glycated and glycated mAb1 fractions. The differences at all individual time points were within 0.3 Da and the total difference summing all time points for all peptides was within 0.8 Da, which indicated that no significant conformational change was induced by glycation based on the criteria described in the method section. Similar results were observed for the light chain (LC) peptides (data shown in Figure S1A). As discussed earlier, the glycated Lys residues in mAb1 were mostly on the surface of the protein. These results indicated that glycation of these Lys residues resulted in structural differences of their side chains, but did not change the conformation of the protein backbone. No changes were detected by CD, AUC or DSC for the glucose-stressed samples (Table S1). Together, these results indicated that glycation had no detectable effects on the secondary structure, tertiary structure or thermostability of mAb1.

Next, HDX MS was used to map the antigen binding sites for mAb1. As shown in Fig. 7B, significant decreases in the HDX levels were observed in 3 regions of the mAb1 HC in the presence of antigen (HC 23–33, HC 47–59 and HC 94–109).

Table 4. Bioassay results for mAb1 DS, non-glycated fraction, glycated fraction and glucose stressed samples. Results are reported as percentages of the reference material values.

				Glucose Stress (mM)				
Assay	DS	Non-Glycated Fraction	Glycated Fraction	0	20	50	100	200
Antigen binding	97	104	61	97	96	93	82	81
CDC activity	99	107	82	102	93	91	92	95
ADCC activity	98	99	75	103	99	102	102	100
FcγRI binding	111	109	92	104	101	103	99	99
FcγRIIIa binding	99	108	81	107	103	116	104	102
FcRn binding	108	126	122	132	132	134	130	140
HC Lys100 glycation by peptide mapping	2.1	ND	21.6	1.2	4.7	9.5	12.8	21.8
Total glycation by Intact LC/MS	15.4	3.1	89.9	13.4	25.1	39.8	54.1	75.6

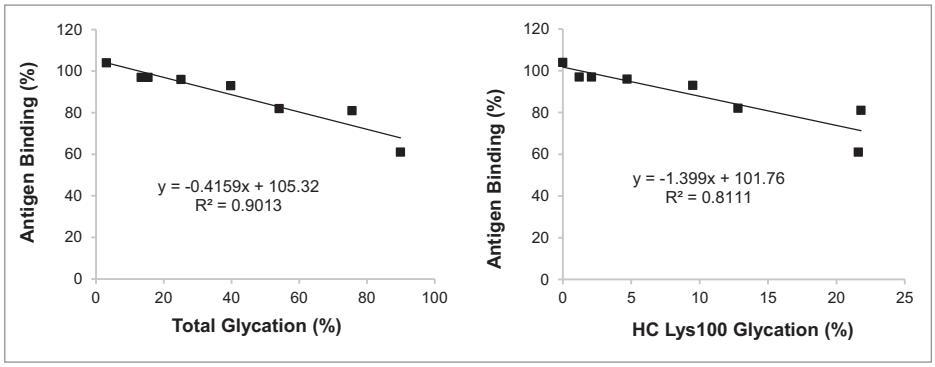


Figure 6. Correlations of antigen binding with the total glycation measured by intact LC-MS (top) and HC Lys100 glycation measured by peptide mapping (bottom).

These 3 regions matched very well with the predicated HC CDRs (CDR-H1 (26–33), CDR-H2 (51–58) and CDR-H3 (98–111)). There was a short peptide (HC 199–202) that also had mild decrease in the HDX levels, which was probably due to the additional conformational changes induced by antigen binding. Similar observations were reported previously.[44,47] Peptides containing HC Lys100, located in CDR-H3, had the most significant changes in HDX levels upon antigen binding, indicating that they were exposed on mAb1 surface but then shielded in the mAb1/antigen complex. For the LC, there were also 3 regions (LC 24–35, LC 47–62 and LC 108–115) that had significant decreases in the HDX levels in the presence of antigen (Figure S1B). The first 2 regions matched very well with the predicated LC CDRs (CDR-L1 (24–34) and CDR-L2 (50–56); however, the third region was slightly away from the predicated CDR-L3 (89–98). This shift could be due to the additional conformational changes induced by antigen binding as discussed above.

Finally, HDX MS was used to evaluate the impact of glycation on antigen binding. Both non-glycated and glycated mAb1 were incubated with antigen and the HDX profiles of the mAb1/antigen complexes were compared to those of the free mAb1 forms. To specifically look at the CDR-H3 binding interface, HDX curves were plotted for the two peptides that contained HC Lys100, HC 94–103 and HC 100–109. As shown in Fig. 7C and 7D, non-glycated peptides 94–103 and 100–109 displayed significant drops in HDX levels in the presence of antigen, which indicated that this region is an antigen binding site. When glycated, these 2 peptides still had drops in HDX levels in the presence of antigen but to a much less extend (Fig. 7E and 7F), which indicated that glycation of HC Lys100 reduced antigen binding. Glycation modified the side chain of HC Lys100, which disrupted the interaction between Lys100 and residues in the antigen. Overall, HDX MS results suggested HC Lys100 glycation did not induce conformational changes in mAb1, but did weaken the binding between mAb1 and antigen, which would explain the bioassay results.

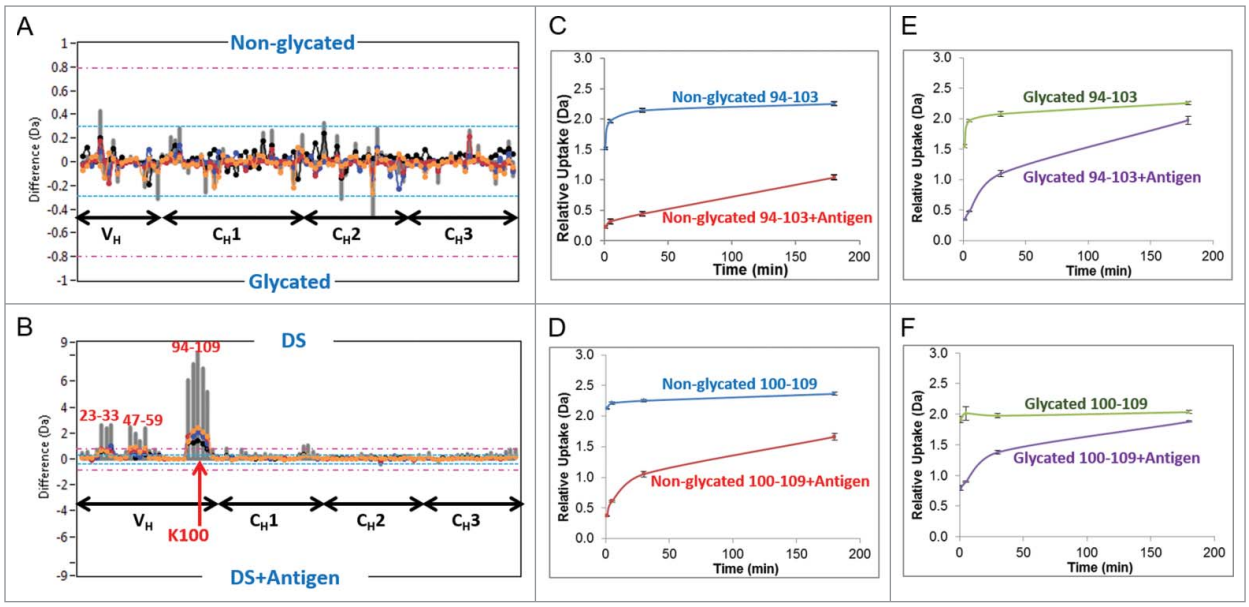


Figure 7. HDX MS analysis of mAb1. A: HDX difference chart comparing the HCs from the non-glycated and glycated mAb1. B: HDX difference chart comparing the HCs from the mAb1 DS and DS+antigen complex. The x-axis represents the peptide number and the data was plotted using the midpoint amino acid position for each peptide. The y-axis represents the HDX difference between the top and bottom protein states (top minus bottom). Colored dots and lines represent differences in deuterium uptake at individual labeling time points (orange = 1 min, red = 5 min, blue = 30 min and black = 180 min). Vertical sticks represent the total HDX differences from all labeling time points. The blue and pink dotted lines represent the criteria for a significant HDX difference for one single labeling time point and for all labeling time points combined, respectively. The location of Lys100 and regions with significant HDX difference are indicated. Comparison of HDX results for glycated and non-glycated peptides 94–103 and 100–109 are presented in C-F. Error bar = 1 SD from triplicate test.

Discussion

Glycation is a common modification of therapeutic antibodies manufactured by the fed-batch process. Our detailed characterization indicated that multiple Lys residues in mAb1 were glycated. HC Lys100 was the most sensitive site and glycation at this residue most likely explains the negative impact of glycation on antigen binding because HC Lys100 is located in the CDR-H3, and HDX-MS analysis showed that glycation of HC Lys100 weakened the antigen binding of mAb1. On the other hand, glycation had little or no detectable impact on the protein conformation or Fc effector functions. These results suggested that glycation of Lys residues in the CDRs of therapeutic antibodies should be carefully characterized.

While the glycation levels in DS samples are typically low, enriching glycated protein using BAC or inducing glycation by glucose stress can help evaluate the impact of glycation. If glycation affects product quality, the level of glycation needs to be monitored and controlled. Both intact LC-MS and peptide mapping are able to quantify glycation levels. Peptide mapping can be used to provide site-specific glycation analysis, but intact LC-MS is a much quicker and simpler method and it provides additional information on the distribution of glycation over the intact molecule. cIEF is a standard assay used for batch release and stability testing, and it could be used as a simple surrogate method for monitoring glycation because the percentage of total acidic peaks or main peak measured by cIEF correlated very well with the levels of glycation measured by intact LC-MS and peptide mapping. Although not all changes in the total acidic peaks or main peak measured by cIEF are due to glycation, based on the acceptance criteria of mAb1 for total acidic peaks or main peak and the linear correlations shown in Fig. 5, the maximal possible level of glycation would be ~24% total glycation or ~4% HC Lys100 glycation even assuming all changes in the total acidic peaks or the main peak are due to glycation. At these levels of glycation, the antigen binding would not be significantly affected (based on correlations in Fig. 6, 24% total glycation or 4% HC Lys100 glycation could lead to 5~10% drop in antigen binding). Therefore, the cIEF specification can ensure the proper control of glycation in this case study.

In summary, our study demonstrated that glycation can be an important quality attribute for monoclonal antibody therapeutics and multiple, orthogonal analytical methods can be used to effectively monitor the levels of glycation.

Materials and methods

Reagents

mAb1 was expressed in CHO cells and manufactured by Janssen Research & Development, LLC. EndoS was purchased from Genovis. Trypsin was purchased from Promega. All other reagents were purchased from Thermo Fisher Scientific or Sigma-Aldrich unless stated otherwise. All reagents were analytical reagent grade or mass spectrometry grade and were used without further purification. All solutions were prepared with Milli-Q water.

Enrichment of Non-glycated and glycated mAb1 by BAC

Non-glycated and glycated mAb1 were separated on a TSKgel Boronate 5PW column (7.5 × 75 mm) from Tosoh. Protein was loaded at 0% B and eluted at 100% B. Mobile phase A was 50 mM EPPS (4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid), 25 mM Tris, 200 mM NaCl, 0.05% NaN3, pH 8.7 and mobile phase B was 500 mM sorbitol, 50 mM EPPS, 25 mM Tris, 200 mM NaCl, 0.05% NaN3, pH 8.7. The eluted non-glycated and glycated fractions were concentrated to ~10 mg/mL (determined by UV absorbance at 280 nm and the Beer-Lambert equation) in mAb1 formulation buffer (25 mM sodium acetate, 60 mM sodium chloride, 140 mM mannitol, 0.04% (w/v) polysorbate 20, pH 5.5).

Glucose Stressed mAb1

mAb1 DS (20 mg/mL) was diluted to 12 mg/mL with 250 mM sodium bicarbonate (pH 8.3) containing glucose at concentrations of 0, 20, 50, 100 and 200 mM, incubated at 37°C for 24 hours, and buffer exchanged through molecular weight cut-off filter to formulation buffer to remove glucose prior to analysis.

Intact LC-MS analysis

Samples were deglycosylated for 1 hour at 37°C with EndoS at 0.5 U/ug of protein. The treated samples were analyzed by LC-MS on a Waters H-class Acquity with a reversed phase C4 column coupled to a Synapt G2-S mass spectrometer. Data were collected and analyzed by MassLynx software. Raw spectra were processed through MaxEnt 1 deconvolution and centered to get the intensities of each peaks.

Peptide mapping analysis

Each sample was denatured with 6 M guanidine-HCl, 100 mM Tris-HCl, and 2.5 mM EDTA, pH 8.0 for 60 minutes at 37°C. Samples were then reduced with 25 mM dithiothreitol (DTT) at 37°C for 1 hour, followed by alkylation with 60 mM sodium iodoacetate at room temperature for 1 hour in the dark. The samples were buffer exchanged into the digestion buffer (50 mM Tris, pH 8.0) using a NAP-5 column (GE Healthcare) before adding Asp-N at a 1:200 (enzyme:protein) ratio for digestion at 37°C for 4 hours. The digested samples were then analyzed by LC-MS/MS (collision-induced dissociation) using an Agilent 1100 HPLC coupled to an ion trap mass spectrometer (Thermo LTQ). Peptide mapping data were analyzed using the Thermo Xcalibur software.

cIEF

Samples were pre-treated with carboxypeptidase B (1:20 (w:w) CPB:protein ratio and incubated at room temperature for 30 min) to remove C-terminal Lys before analysis on a Protein Simple iCE280 Analyzer using a 100-μm inner wall-coated silica capillary without an outer wall polyimide coating, an anolyte solution of dilute phosphoric acid and methylcellulose, and a catholyte solution of sodium hydroxide and methylcellulose. pI markers of 7.40 and 9.46 from Protein Simple were used for calibration. Protein was detected at UV 280 nm.

Bioassays

Antigen and Fc receptor (FcγRI, FcγRIIIa and FcRn) binding activities were measured using the competitive time-resolved fluorescence resonance energy transfer (TR-FRET) assays (LANCE reagents from Perkin Elmer). mAb1 samples at different concentration were incubated with the antigen/antibody or Fc receptor/antibody complexes to induce changes in the fluorescence signals and the fluorescence signals were plotted against sample concentration and analyzed by a 4-parameter curve fit. The concentration (EC50) needed to reach 50% of the maximum TR-FRET signal was calculated for each sample and the relative binding activity of each sample was calculated from the ratio of the EC50 of reference and the tested samples, and expressed as a percentage relative to reference material.

ADCC activity was measured using a commercially available gene reporter kit (Bio-Glo™). FcγRIIIa receptors expressed on the engineered effector cells recognized mAb1 that bound on the target cells and triggered the NFAT (nuclear factor of activated T-cells) pathway for firefly luciferase expression. The luciferase activity in the effector cells was quantified by measuring the luminescence, which was related to the degree of FcγRIIIa receptor binding by the antibody (mAb1). The signal obtained by a microtiter luminescence plate reader was plotted against sample concentrations and analyzed by a 4-parameter curve fit. The ADCC activity of the sample was calculated as a ratio of EC50 of the samples relative to the EC50 of the reference material and expressed as a percentage.

CDC was measured by incubating target cells with the mAb1 samples, followed by the addition of human complement in normal serum. The Fab portion of mAb1 bound to the antigen on the surface of target cells and the Fc portion of mAb1 bound to the complement in normal serum, leading to activation of the complement cascade and cell lysis. The concentration of viable cells was measured using the Luminescent Cell Viability commercial kit (CellTiter-Glo™), which produced luminescence as a function of the amount of ATP present after lysis of the cells. The luminescence measured by a microtiter luminescence plate reader as a function of mAb1 concentration was used to measure the ability of mAb1 to induce cell lysis through the complement system. The signal obtained was plotted against sample concentration and analyzed by a 4-parameter curve fit. The CDC activity of the test sample was calculated as a ratio of EC50 of the samples relative to the EC50 of the reference material and expressed as a percentage.

HDX MS

The mAb1/antigen complex was generated by mixing mAb1 (glycated or non-glycated) with antigen at a molar ratio of 1:2 (mAb1:antigen). HDX MS analysis was performed using a Waters 2D nanoAcquity UPLC HDX system coupled with a Synapt G2-S mass spectrometer as described previously.[44] Briefly, samples were diluted 15-fold into labeling buffer (5 mM K_2HPO_4 and 5 mM in KH_2PO_4 in D_2O, pD 7.0) and the exchange reaction was maintained at 20°C for increasing times between 1 and 180 min. pD was calculated by adding 0.4 unit to the pH meter reading.[49] The reaction was then stopped by diluting samples 1:1 (v:v) with the quenching buffer (4 M guanidine HCl, 50 mM K_2HPO_4, 50 mM in KH_2PO_4 and 0.5 M TCEP, pH 2.5) at 1°C. The quenched samples were injected into the Waters HDX system, where they were digested by flowing through an online immobilized pepsin column (Applied Biosystems, 2.1 × 30 mm), desalted on a Waters VanGuard precolumn (2.1 × 5 mm), separated on a reversed phase UPLC column (Waters Acquity BEH300 column, 1.0 × 100 mm) and injected into the mass spectrometer. The peptides resulting from online pepsin digestion were identified by running a separate experiment of the mAb1 control sample without deuterium labeling and analyzed using the Waters ProteinLynx Global Server (PLGS). The extent of deuterium uptake for each peptide at different time points were calculated using the DynamX software. HDX difference charts were plotted to compare any two protein states, and statistical analysis () was used to set up the criteria based on intermediate precision, which was calculated as the weighted 3SD from 3 technical replicates of a standard mAb that were run on three different days.[44,50] For our HDX MS system, peptides with results that met the following two criteria simultaneously were considered statistically significant with a 99.7% confidence interval: 1) the measured individual HDX difference was greater than 0.3 Da for at least one labeling time point; and 2) the total HDX difference summed from five time points was greater than 0.8 Da.

Abbreviations

ADCC	antibody-dependent cell-meditated cytotoxicity
AUC	analytical ultracentrifugation
BAC	boronate affinity chromatography
CD	circular dichroism
CDC	complement-dependent cytotoxicity
CDR	complementary-determining region
CHO	chines hamster ovary
cIEF	capillary isoelectric focusing
CPB	carboxypeptidase B
DS	drug substance
DSC	differential scanning calorimetry
FcRn	neonatal Fc receptor
HDX	hydrogen/deuterium exchange
LC	liquid chromatography
Lys	lysine
mAb	monoclonal antibodies
MS	mass spectrometry

Disclosure of potential conflicts of interest

The authors declare that there are no conflicts-of-interest and they have no financial relationship that would require disclosure in connection with the work presented in this paper.

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