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Characterization of the glycosylation state of a recombinant monoclonal antibody using weak cation exchange chromatography and mass spectrometry

Georgeen Gaza-Bulseco, Ashley Bulseco, Chris Chumsae, Hongcheng Liu *

Process Sciences Department, Abbott Bioresearch Center, 100 Research Center, Worcester, MA 01605, United States

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Abstract

Recombinant monoclonal antibody heterogeneity is inherent due to various enzymatic and non-enzymatic modifications. In this study, a recombinant humanized monoclonal IgG1 antibody with different states of glycosylation on the conserved asparagine residue in the CH_2 domain was analyzed by weak cation exchange chromatography. Two major peaks were observed and were further characterized by enzymatic digestion and mass spectrometry. It was found that this recombinant monoclonal antibody contained three glycosylation states of antibody with zero, one or two glycosylated heavy chains. The peak that eluted earlier on the cation exchange column contained antibodies with two glycosylated heavy chains containing fucosylated biantennary complex oligosaccharides with zero, one or two terminal galactose residues. The peak that eluted later from the column contained antibodies with either zero, one or two glycosylated heavy chains. The oligosaccharide on the antibodies eluted in the later peak was composed of only two GlcNAc residues. These results indicate that conformational changes in large proteins such as monoclonal antibodies, caused by different types of neutral oligosaccharides as well as the absence of oligosaccharides, can be differentiated by cation exchange column chromatography.

Keywords: Recombinant monoclonal antibody; Oligosaccharides; WCX-10; Mass spectrometry

1. Introduction

A recombinant monoclonal antibody is synthesized based on specific gene sequences for both light and heavy chains, which should result in the production of a homogeneous population of molecules with the same protein sequence. However, enzymatic and non-enzymatic modifications introduced during protein synthesis and assembly, cell culture, purification, formulation, storage and incubation under various accelerated stability conditions often convert the homogeneous population into a heterogeneous one. Thus antibody products do demonstrate heterogeneity in characteristics such as molecular weight, charge, and conformation.

Ion exchange chromatography is a method commonly used to analyze antibody charge heterogeneity. When analyzed by ion-exchange chromatography, monoclonal antibodies usually elute in multiple peaks. Multiple peaks of different retention times have been determined to be due to factors such as the absence or presence of a C-terminal lysine (Lys) residue [1–8], cyclyzation of the N-terminal glutamine to pyroglutamate [2,7], deamidation [5,7,9–12], the presence of sialic acid [3,7], isomerization [11], amidation [8], expression of a leader or intron sequence [6,7], or oxidation of methionine residues [13].

The effect of various modifications on the elution of antibodies from ion exchange columns can be classified into three categories including direct contribution to charge difference, positional effect and conformational effect. Firstly, modifications can affect the elution of antibodies from ion exchange chromatography columns by contribution to charge directly. For example, a Lys residue introduces a positive charge. Therefore, on cation exchange columns antibodies with two C-terminal Lys will elute later than antibodies with one C-terminal Lys, followed by antibodies containing no C-terminal Lys. Treatment with carboxypeptidase B, which removes C-terminal Lys, changes this profile of three peaks with different Lys residues into a single peak containing antibodies with no C-terminal

* Corresponding author. Tel.: +1 508 849 2591; fax: +1 508 793 4885.
E-mail address: Hongcheng.liu@abbott.com (H. Liu).

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Lys residues [1,3,9]. This indicates that the presence of the C-terminal Lys affects the elution profile of antibodies from ion exchange columns only by direct alteration of charge. The effect of sialic acid on elution is also based on net charge differences. Sialic acid introduces a negative charge and antibodies with oligosaccharides containing sialic acid will elute earlier on cation exchange columns. Removal of sialic acid with sialidase treatment results in elution of antibodies consistent with antibodies containing asialylated oligosaccharides [3]. Secondly, the same modification located at different positions of the antibody can have an effect on the retention time. For example, antibodies with the same number of pyroglutamate residues elute at different retention times depending on the position of the pyroglutamate [2]. Positional effects on elution profile have also been reported for deamidation [9]. The positional effects are likely due to the fact that antibodies are not perfectly symmetrical as demonstrated by Sapphire et al. [14,15], which would lead to differences in local surface charges that interact with the column matrix. Lastly, modifications can affect elution by affecting conformation. This is shown by comparing the retention times of antibodies with either aspartate or isoasparate residues located at the same position. From the chemical point of view, isoaspartate is slightly more acidic than aspartate and the antibody containing isoaspartate would be expected to elute earlier from a cation exchange column. However, antibodies with aspartate elute earlier than antibodies with isoaspartate [11]. Isoaspartate introduces an additional methane group to the peptide backbone compared to aspartate, therefore it may cause more significant structural changes than aspartate to the antibody. Although the effects of modifications on column retention time can be classified, in reality, chromatographic behavior of monoclonal antibodies is likely due to simultaneous contribution of multiple factors. Therefore, the behavior of monoclonal antibodies on column chromatography is not always predictable based on the chemical nature of the modifications alone.

In this study, a recombinant humanized monoclonal antibody produced in human embryonic kidney (HEK) 293 cell line during the research stage was analyzed by weak cation exchange chromatography. The major peaks were analyzed by enzymatic digestion and mass spectrometry to determine their composition. It was found that the multiple peaks were due to different glycosylation states of the antibody.

2. Experimental

The recombinant humanized monoclonal IgG1 antibody was expressed in HEK 293 cell line by transient expression and purified using protein A chromatography at Abbott Bioresearch Center (Worcester, MA).

A Shimadzu HPLC and a weak cation exchange WCX-10 column (250 mm × 4 mm) from Dionex (Sunnyvale, CA) were used for analyzing charge variants of this antibody. Mobile phase A was 10 mM sodium phosphate, pH 6.0. Mobile phase B was 10 mM sodium phosphate, 500 mM sodium chloride, pH 6.0. The samples were injected at 90% mobile phase A and 10% mobile phase B. After running at the initial condition for 5 min, the percentage of mobile phase B was increased to 50% within 20 min. The column was then washed by increasing mobile phase B to 100% within 5 min and running at 100% mobile phase B for 5 min. The percentage of mobile phase B was decreased to 5% in 5 min. The column was equilibrated at 5% mobile phase B for 10 min before the next injection. The flow-rate was set at 1 mL/min. Protein elution was monitored at UV 280 nm and 214 nm. Results were reported in mV and the instrument was set at 2.5 AU/V.

Fractions of the recombinant monoclonal antibody were collected using a semi-preparative WCX-10 column (250 mm × 9 mm) from Dionex. The mobile phases and gradient were the same as described previously for the analytical WCX-10 column except a 5 mL/min flow rate was used. The fractions were collected and concentrated using Amicon Ultra-4 centrifugal filter devices (Millipore, Billerica, MA) with a molecular weight cut-off of 10 kDa. The purity of the fractions was analyzed using the analytical WCX-10 column procedure.

For deglycosylation, the recombinant monoclonal antibody and the collected fractions were digested with PNGaseF (2.5 mU/μL, Prozyme, San Leandro, CA) at a ratio of approximately 1 μL enzyme: 200 μg antibody. *N*-Octylglucoside (Roche, Indianapolis, IN) was included in the sample preparation to a final concentration of 1% (w/v) to facilitate the removal of N-linked oligosaccharides. Digestion was allowed to proceed at 37 °C for 18 h. The samples were reanalyzed using analytical WCX-10 chromatography, and fractions were collected using the semipreparative WCX-10 column.

For molecular weight determination, the samples were first diluted to 0.5 mg/mL and then reduced with 10 mM dithiothreitol (DTT) (Sigma, St. Louis, MO) at 37 °C for 30 min. Molecular weights of the reduced samples were determined by LC–MS. An Agilent HPLC (Santa Clara, CA) and a protein C4 column (Vydac, 150 mm × 1 mm i.d., 5 μm particle size, 300 A pore size) were used to desalt, separate and introduce samples into a Q star mass spectrometer (Applied Biosystems, Framingham). Five micrograms of each sample was loaded at 95% mobile phase C (0.02% TFA, 0.08% FA in Milli-Q water) and 5% mobile phase D (0.02% TFA, 0.08% FA in acetonitrile). After running at 5% mobile phase D for 5 min, proteins were eluted off the column by increasing mobile phase D to 65% within 35 min. The column was washed by increasing mobile phase D to 95% in 5 min and then decreasing to 5% in another 5 min. The column was equilibrated at 5% mobile phase D for 10 min before the next injection. The flow rate was set at 50 μL/min and the column oven was set at 60 °C. The mass spectrometer scan range was set at a range of *m/z* 800–2500. The IonSpray voltage was set at 4500 V and the source temperature was set at 350 °C.

For peptide map, fractions of the deglycosylated antibody were denatured with 6 M guanidine hydrochloride in 100 mM Tris, pH 8.0, reduced with 10 mM DTT at 37 °C for 30 min and then alkylated with 25 mM iodoacetic acid (Sigma) at 37 °C for another 30 min. The samples were diluted six fold using Milli-Q water. Trypsin (Worthington, Lakewood, NJ) was added to the samples at an enzyme:antibody ratio of 1:50 (w/w) and the samples were incubated at 37 °C for 18 h. Proteolysis was stopped by adding 1N HCl. The Agilent HPLC and a C18 column (Vydac, 250 mm × 1 mm i.d., 5 μm particle size, 300 A pore

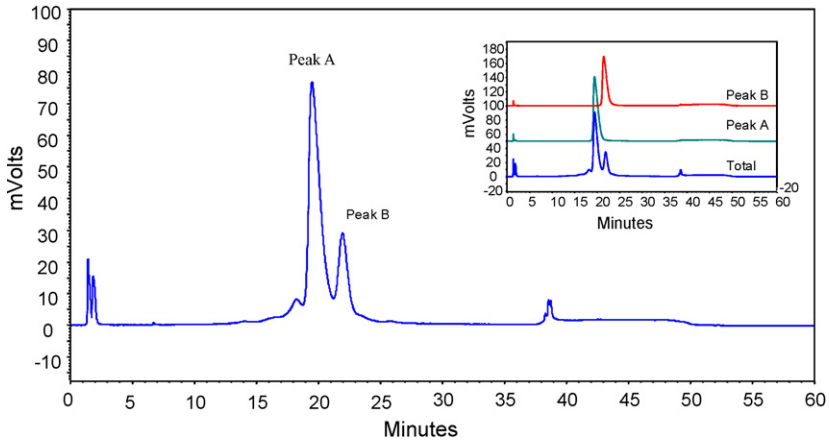

Fig. 1. WCX-10 chromatogram of a recombinant monoclonal antibody. Peaks A and B are the two major peaks. Material eluted before peak A is referred to as acidic species. Peaks A and B were collected and reanalyzed by WCX-10 (inset) to show the purity.

size) were used to separate and introduce peptides into the Q Star mass spectrometer. Approximately 20 μg of each sample was loaded at 98% mobile phase A and 2% mobile phase B, then eluted by increasing mobile phase B from 2 to 35% in 140 min. The flow rate was set at 50 μL/min and the IonSpray voltage was set at 4200 V. Source temperature was set at 75 °C and *m/z* was scanned from 250 to 2000.

3. Results

3.1. WCX-10 chromatography

A typical WCX-10 chromatogram is shown in Fig. 1. Two major peaks (A and B) at retention times of approximately 20 and 22 min were observed, which accounted for 64 and 24% of the total peak area, respectively. In addition, approximately 12% of this antibody eluted before peak A, which was referred to as acidic species as they eluted earlier than the main peak. This study focused only on the characterization of peaks A and B.

3.2. LC–MS analysis of peaks A and B

In order to determine the difference between peaks A and B, fractions from these peaks were collected, concentrated and analyzed by LC–MS. As shown in the inset of Fig. 1, the collected fractions eluted at the same retention times as that of peaks A and B in the initial sample, which suggested that sample manipulation did not introduce significant changes to the antibody.

The fractions of peaks A and B were analyzed by LC–MS. As shown in Fig. 2A, three major peaks in the mass spectrum were observed in peak A with molecular weights of 51,109, 51,271, and 51,433 Da, which are in good agreement with the calculated molecular weights of the heavy chain with oligosaccharides Gal 0 (51,106 Da), Gal 1(51,272 Da) or Gal 2 (51,434 Da), respectively. On the other hand, two principal mass components were observed for peak B (Fig. 2B) with molecular weights of 49,663 and 50,070 Da. The molecular weight of 49,663 Da corresponded to the calculated molecular weight (49,661 Da) of the heavy chain without oligosaccharides, while the molecular weight of 50,070 Da corresponded to the heavy chain with a disaccharide consisting of two GlcNAc units (50,068 Da). The peak identities were therefore determined based on the molecular weights. Peak A contained antibody with two glycosylated heavy chains with typical oligosaccharides observed for recombinant monoclonal antibodies expressed in mammalian cell cultures [16–19]. Peak B contained antibodies with either, zero, one or two non-glycosylated heavy chains. The glycosylated heavy chain in peak B contained a disaccharide, GlcNAcGlcNAc.

3.3. PNGaseF treatment and WCX-10 analysis

To confirm the state of glycosylation of peak A and to better understand the nature of peak B, the antibody was digested with PNGaseF and analyzed by weak cation exchange chro-

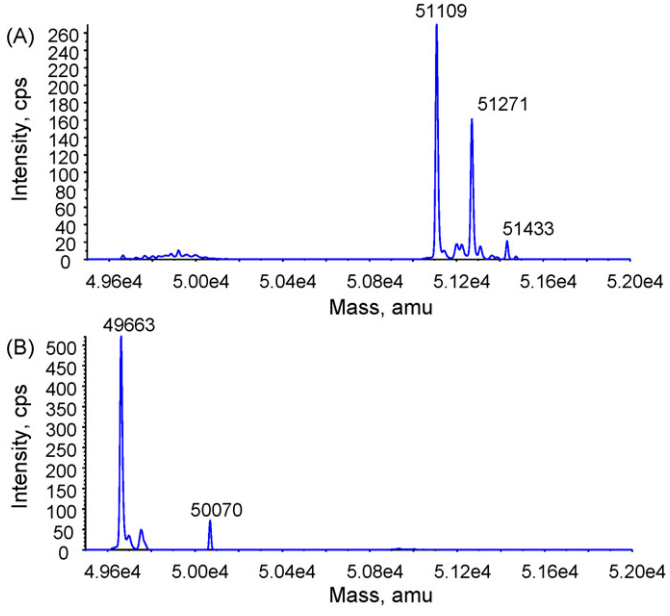

Fig. 2. Mass spectra of the heavy chain from peak A (A) and peak B (B).

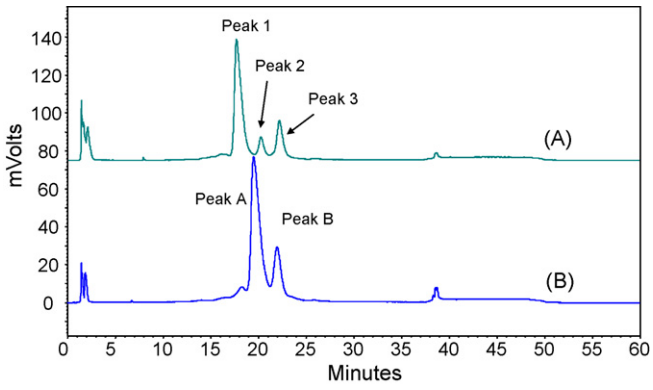

Fig. 3. WCX-10 chromatograms of the recombinant monoclonal antibody before (B) and after (A) PNGaseF treatment. Peaks A and B are the two major peaks before PNGaseF treatment. After PNGaseF treatment, three major peaks, 1–3, were observed.

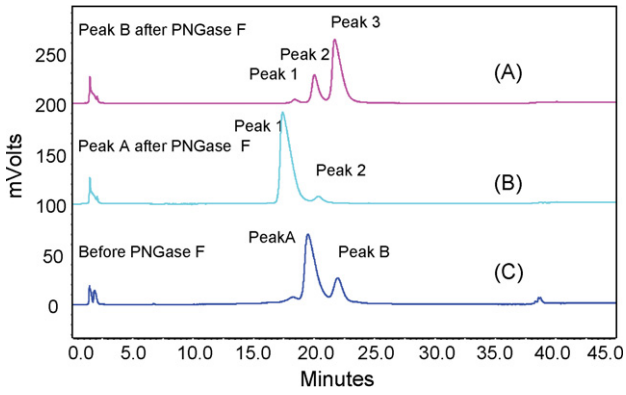

Fig. 4. WCX-10 chromatograms of peaks A and B after PNGaseF treatment. (A) Peaks 1–3 were generated from PNGaseF treatment of peak B. (B) Peaks 1 and 2 were generated from PNGaseF treatment of peak A. (C) The recombinant monoclonal antibody before PNGaseF treatment.

matography using a WCX-10 column. Digestion by PNGaseF removes the N-linked oligosaccharides and converts the original asparagine residue to an aspartate residue, which introduces one negative charge and should result in earlier elution from the WCX-10 column. As shown in Fig. 3A, after PNGaseF treatment, three peaks (peaks 1–3) were observed. Peak 1 was the major peak, which had an earlier retention time than peak A, before deglycosylation. This observation confirmed that peak A contained mainly antibodies with two glycosylated heavy chains. The removal of oligosaccharides from the two heavy chains generated two aspartate residues and resulted in an earlier elution due to the net negative charge increase. Peak 3, on the other hand, had the same retention time as peak B in the sample before deglycosylation, which suggested that peak B contained mainly antibodies with two non-glycosylated heavy chains. Peak 2, which eluted between peaks 1 and 3, was thus hypothesized to result from the deglycosylation of the antibodies with one glycosylated heavy chain and one nonglycosylated heavy chain. Deglycosylation converted the asparagine residue with oligosaccharides into aspartate, while the asparagine residue on the non-glycosylated remained unchanged.

The individual fractions of peaks A and B were also digested using PNGaseF and analyzed by WCX-10 chromatography. As shown in Fig. 4, digestion of peak A resulted in the generation of mainly peak 1 (Fig. 4B). A small peak with the same retention as peak 2 was also observed. The origin of this peak was unclear, but it could be antibodies with one glycosylated heavy chain that coeluted with peak A before deglycosylation or contamination from peak B during fraction collection. Digestion of peak B resulted in the generation of peaks 1–3 (Fig. 4A). Based on these results, the fully glycosylated antibody eluted earlier from the column in peak A and the non-glycosylated and partially glycosylated antibodies eluted later in peak B. It was hypothesized that peak 1 contained two aspartate residues on the two heavy chains resulting from deglycosylation of the two glycosylated heavy chains. Peak 2 contained antibodies with one aspartate residue resulting from deglycosylation of the glycosylated heavy chain and one asparagine residue resulting from nonglycosylated heavy chain. Peak 3 contained antibodies containing two asparagine residues of the two non-glycosylated heavy chains.

3.4. Peptide map analysis of peaks 1–3

To determine their composition, peaks 1–3 were collected and analyzed by tryptic peptide mapping. Searching for the molecular weights of the peptide containing the conserved asparagine (EEQYNSTYR) with either asparatate ($MH^+ = 1189.5$ Da) or asparagine ($MH^+ = 1188.5$ Da) residues resulted in one peak for peak 1, two peaks for peak 2 and one peak for peak 3 as shown in the TIC chromatograms in Fig. 5. The molecular weights of the different peaks can be calculated from the doubly charged monoisotopic peaks as shown as insets of Fig. 5. The molecular weight of the peak derived from peak 1 was 1189.6 Da, which is in agreement with the molecular weight of the peptide with an aspartate residue instead of an asparagine residue. The molecular weight of the peak from peak 3 was 1188.6, which is in good agreement with the peptide with an asparagine residue. The molecular weight of the first peak from peak 2 was 1189.6 and the second peak was 1188.6 Da, which corresponded to peptides with aspartate and asparagine residues, respectively.

Taken together, as summarized in Table 1, peak A contained antibody with two glycosylated heavy chains with

Table 1
Identities of peaks shown in Fig. 3

Peaks (trace B of Fig. 3)	States of glycosylation	States of Asn	Peaks (trace A of Fig. 3)
	PNGase F treatment		
Peak A			Peak 1
Peak B			Peak 2
			Peak 3

(○) Complex oligosaccharides; (□) $(GlcNAc)_2$.

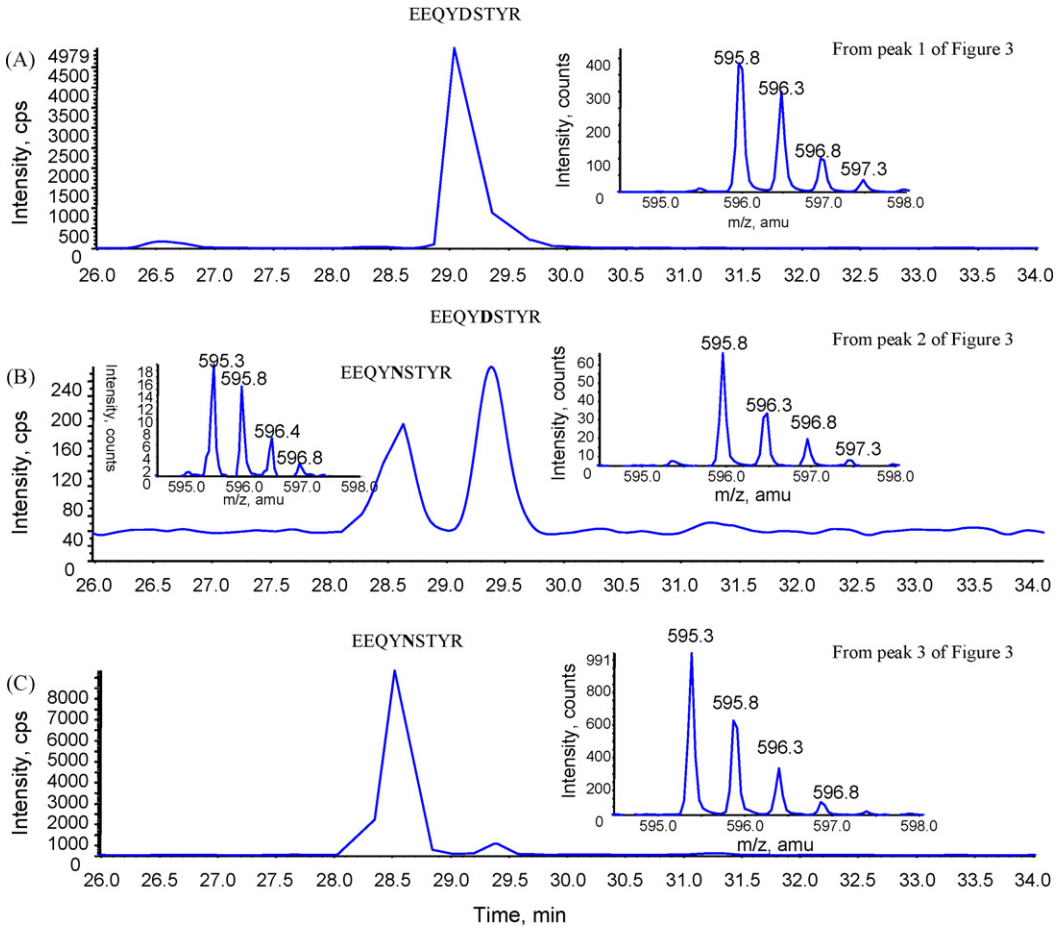

Fig. 5. Total ion current chromatograms of the peptide containing the conserved Asn residue for peaks 1–3 of Fig. 3. The samples were digested with trypsin after PNGaseF treatment. The spectra were obtained from peak 1 (A), peak 2 (B), and peak 3 (C). The doubly charged monoisotopic peaks of each TIC peak are shown as insets.

typical oligosaccharides. Peak B contained antibody with zero (major component), one or two glycosylated heavy chains. The oligosaccharide in peak B material was a disaccharide with two GlcNAc residues. After deglycosylation, peak 1 contained two aspartate residues, peak 2 contained one aspartate and one asparagine residues, and peak 3 contained two asparagine residues in the antibody heavy chain glycosylation site.

4. Discussion

Glycosylation of the conserved asparagine residue is a common posttranslational modification of recombinant monoclonal antibodies. The most common glycoforms are biantennary complex structures with a core fucose and either zero, one or two terminal galactose residues. High-mannose oligosaccharides with five, six, seven or more mannose residues have also been observed. There are several reports on the presence of sialic acid on monoclonal antibodies but to a much lower percentage [7,20–23].

Oligosaccharides affect the elution profile of antibodies from cation exchange chromatography by charge, either directly or indirectly. For example, the presence of sialic acid, which bears a negative charge, results in an earlier elution of the antibodies on cation exchange chromatography [3,7,24], and therefore sialic acid contributes directly to the charge difference of the molecule. On the other hand, oligosaccharides may affect the elution profile by modulating antibody structure and local charge distribution. As in the case presented in this study, nonglycosylated antibodies eluted later from a weak cation exchange column than glycosylated antibodies even though the attached oligosaccharides bear no charge difference. It has been well documented that oligosaccharides and their interactions with the protein moieties are critical for the structural integrity of the antibodies especially within the CH_2 domains [25–31], and antibodies with or without oligosaccharides have different structures. These structural differences can cause surface charge distribution differences, which therefore result in antibodies with different retention times in column chromatography. Furthermore, antibodies with different forms of oligosaccharides adopt different conformations as demonstrated by X-ray crystallography [25] and differential scanning calorimetry studies [29]. These studies showed that smaller oligosaccharides resulted in antibodies with structures most similar to that of nonglycosylated antibodies. Therefore, antibodies with one or two glycosylated heavy chains containing only two GlcNAc residues will have a structure more similar to antibodies with two non-glycosylated heavy chains and will therefore coelute from the weak cation exchange column.

The recombinant monoclonal antibody transiently expressed in a HEK 293 cell line produced a mixed population of antibodies containing zero, one or two glycosylated heavy chains. In addition to the typical oligosaccharides, an oligosaccharide containing only two GlcNAc residues was also found attached to heavy chains. N-linked oligosaccharides are transferred to the protein moiety as a unit of a high mannose type sugar [32] that is processed by multiple enzymes in the endoplasmic reticulum and Golgi to produce oligosaccharides with the same core structure with a minimum of five sugar residues ($(GlcNAc)_2(Man)_3$). It was therefore unusual to find antibodies with heavy chains containing only two GlcNAc residues. The presence of an oligosaccharide on the heavy chain containing only two GlcNAc residues could be due either to degradation of the oligosaccharides or a defect in oligosaccharide synthesis process. Degradation of the oligosaccharide seems unlikely because no other oligosaccharide intermediates between the disaccharide and NGA2F glycan were observed. Therefore, it seems more likely that some of the HEK 293 cells contained deficient oligosaccharide synthesis systems, which resulted in antibodies without oligosaccharides, and more interestingly, heavy chains containing only two GlcNAc residues.

In this study, a recombinant monoclonal antibody was expressed in HEK 293 cell line and resulted in a mixed population of antibodies with zero, one or two glycosylated heavy chains. We showed that it was possible to resolve the different glycosylation states of the antibody by weak cation exchange chromatography. Cation exchange chromatography is usually used to characterize the charge heterogeneity of monoclonal antibodies. In theory, it separates antibody variants based on the net charge differences. However, it can also recognize local charge differences caused by the absence of oligosaccharides and the presence of different types of neutral oligosaccharides as demonstrated in the current study. This information would be highly beneficial for column chromatography method development and antibody characterization.

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