

# In-Depth Method for the Characterization of Glycosylation in Manufactured Recombinant Monoclonal Antibody Drugs

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## S Supporting Information

**ABSTRACT:** The glycosylation in recombinant monoclonal antibody (rMab) drugs is a major concern in the biopharmaceutical industry as it impacts the drugs' many attributes. Characterization is important but complicated by the intricate structures, microheterogeneity, and the limitations of current tools for structural analysis. In this study, we developed a liquid chromatography–mass spectrometry (LC–MS) N-glycan library based on eight commercial rMab drugs. A library of over 70 structures was developed for the rapid characterization of rMab. N-Glycans were separated on a porous graphitized carbon (PGC) column incorporated on a chip and then analyzed by an electrospray ionization hybrid quadrupole time-of-flight (ESI-Q-TOF) MS. The retention time and accurate mass for each N-glycan were recorded in the library. The complete structures were obtained through exoglycosidase sequencing. The results showed that most of the N-glycans between different antibodies are nearly the same with different abundances. The utility of this library enables one to identify structures in a rapid manner by matching LC retention times and accurate masses.

No.	Name	RT	Mass	Structure
1	N34100a	17.56	1464.56	
2	N44100a	18.51	1626.65	
3	N44100b	18.91	1626.65	
4	N34000a	15.15	1318.51	
5	N54100a	19.98	1788.67	
6	N33100b	17.13	1261.48	
7	N33100a	17.61	1261.48	
8	N43100b	18.34	1423.57	

Recombinant monoclonal antibody (rMab) drugs have emerged as a powerful class of biopharmaceuticals. Their specificity toward target antigens makes them effective treatments for cancers and other chronic diseases.<sup>1–4</sup> rMab-based drugs have multiple functions including blocking the signal transduction of tumor cells to proliferate, activating the immune system to kill tumor cells, and carrying cancer drugs or radiation targets to tumor cells.<sup>3</sup> The first licensed rMab drug was muromonab in 1986, and it is estimated that approximately 30% of the new drugs approved in the next decade will be rMab drugs.<sup>1,3,5–8</sup> Currently, there are more than 30 approved rMab drugs and hundreds of new rMab drug candidates under clinical trials.<sup>5</sup> All currently approved rMab drugs are based on IgG and are usually manufactured from Chinese hamster ovary (CHO), mouse myeloma NS0, and mouse myeloma Sp2/0 cell lines.<sup>1,3,5–10</sup>

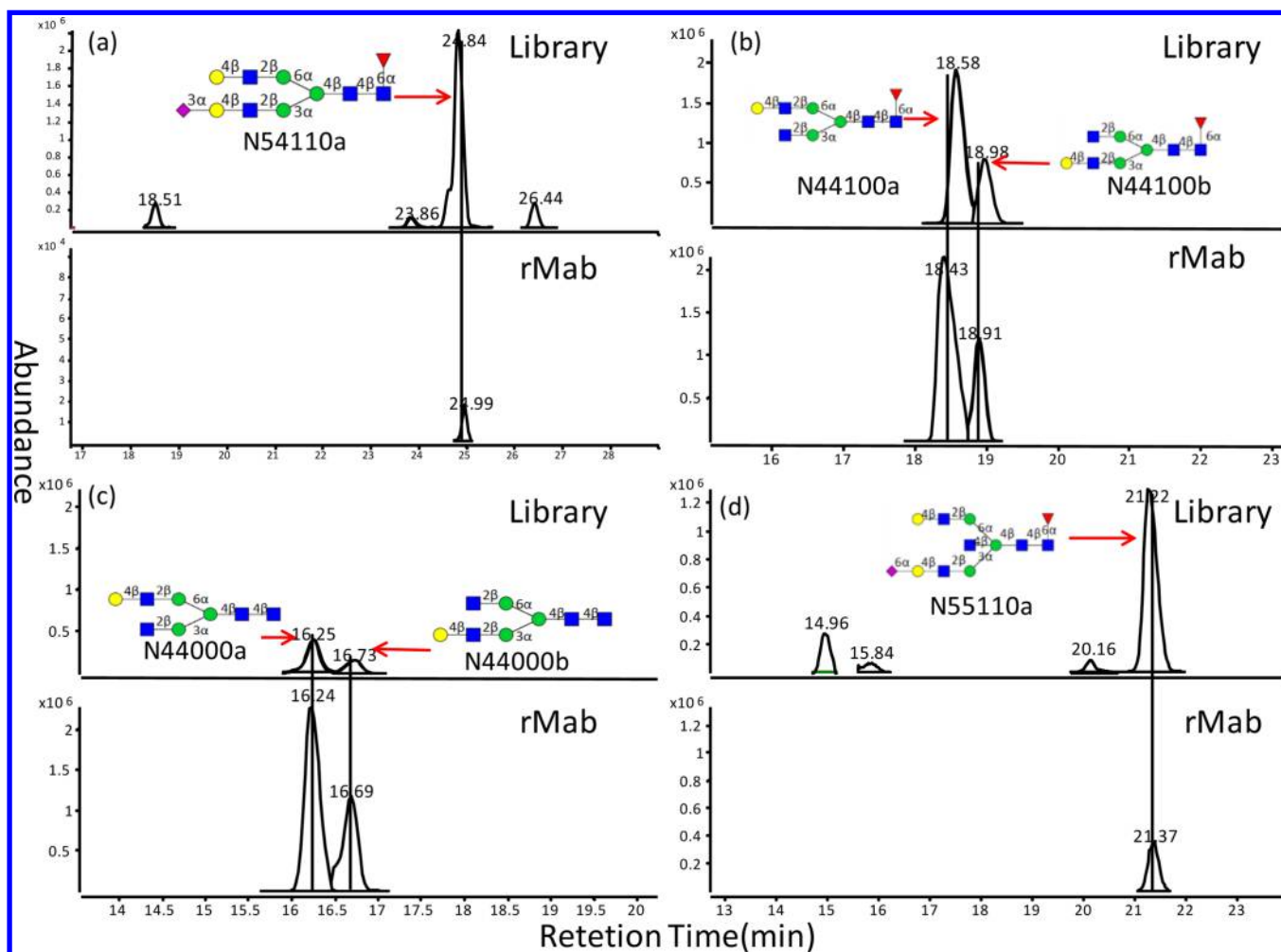
rMab is composed of two identical light chains and two identical heavy chains. The antibody contains three domains: the antigen binding (Fab), the hinge region, and the fragment crystallizable (Fc) domain.<sup>9,10</sup> There is one N-glycosylation site (Asn 297) found in the Fc region on both identical heavy chains. Some rMabs may contain oligosaccharides in the Fab region.<sup>11,12</sup> Although the oligosaccharides in the Fc region only account for 2–3% of the total protein mass, they have a major affect in functions related to drug efficacy, pharmacokinetics, stability, and immunogenicity.<sup>10,13–15</sup> For example, the effector function of the deglycosylated or aglycosylated rMab is found to be severely compromised or ablated,<sup>15</sup> while an increased level of N-glycolylneuraminic acid (NeuGc) produces shorter half-life.<sup>16</sup> In general, specific glycoforms can increase or decrease the drug efficacy, although the precise reason may not always be clearly understood.<sup>14,15,17</sup> Therefore, deep structural characterization of N-glycosylation of rMab is essential for drug

development and production. However, the microheterogeneity of the glycans, their diverse compositions, the large number of isomeric structures, and the large variations in abundances all make extensive glycan characterization of rMab a slow and tedious process.<sup>18,19</sup> Furthermore, because the best known pharmaceuticals were discovered much earlier when glycoanalytics was not as advanced, the threshold for characterization was much lower. Glycan analysis was focused on three components known as G0F, G1F, and G2F.<sup>20,21</sup> However, the coming tide of biosimilars and follow-on biologics will require considerably better glycan characterization.

In this report, we describe a method for the rapid characterization of rMab glycosylation using a detailed N-glycan library exclusively for rMab. The library was constructed using commercial rMab drugs including trastuzumab, bevacizumab, rituximab, cetuximab, panitumumab, infliximab, ofatumumab, and eculizumab. NanoLC-Chip-Q-TOF analysis, exoglycosidase sequencing, and a reference N-glycan library built from human serum glycoproteins were used to build the rMab N-glycan library.<sup>22,23</sup> All the N-glycans were separated on the nanoLC-chip with a PGC column. Owing to the excellent separation performance of the PGC medium and the high mass accuracy of TOF MS, each N-glycan isomer has a unique retention time and accurate mass.<sup>24–27</sup> The reproducible retention time and accurate mass are used to rapidly identify unknown glycans in the rMab.<sup>25,28–30</sup>

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**Figure 1.** Illustration of the identification procedure for antibody N-glycans using a previously annotated reference library. (a) Extracted compound chromatogram (ECC) of composition N54110 from an N-glycan reference library and from the rMab. Four isomers were found in the reference library for the monofucosylated monosialylated structure. On the basis of the similarities in accurate mass and retention times, the structure was determined as shown (inset). (b) Two isomers with similar retention times corresponding to monofucosylated biantennary structures. The rMab glycans matched those corresponding to the library and the structures were determined (inset). (c) Two isomers corresponding to terminal monogalactosylated structures. The structures were determined and are shown (inset). (d) A bisecting GlcNAc with monosialylated and monofucosylated structure as determined by comparison to the reference library. Compounds were reduced to the alditol prior to the LC–MS analysis. Symbolic N-glycan structures correspond to (blue ■) N-acetylglucosamine, (green ●) mannose, (yellow ●) galactose, (○) hexose, (red ▲) fucose, (purple ◆) N-acetyl neuraminic acid, (◇) N-glycolylneuraminic acid. Linkages are provided at the glycosidic bond.

## EXPERIMENTAL SECTION

**Chemicals and Reagents.** All the rMab drugs were obtained from UC Davis Medical Center. Peptide, N-Glycosidase F (PNGase F), and exoglycosidases,  $\alpha$ (2-3)-neuraminidase (sialidase),  $\alpha$ (1-2,3)mannosidase, and  $\beta$ -N-acetyl glucosaminidase ( $\beta$ -GlcNAcase), were obtained from New England Biolabs (Ipswich, MA);  $\beta$ (1-4)Galactosidase and  $\alpha$ (1-3,4)fucosidase were obtained from Prozyme (Hayward, CA). Dithiothreitol (DTT) was purchased from Promega (Madison, WI). Sodium borohydride was purchased from Sigma-Aldrich (St. Louis, MO). All reagents are of analytical or HPLC grade.

**N-Glycan Release, Reduction, and Purification.** N-glycans were released from rMab using standard PNGase F methods.<sup>31</sup> Briefly, rMab drugs were first denatured by DTT and then treated with PNGase F to release N-glycans. Free N-glycans were then purified by solid phase extraction (SPE) using graphitized carbon cartridges (GCC) (Alltech Associated, Deerfield, IL) in an automated manner using a Gilson GX-274

ASPEC robot liquid handler. Purified N-glycans were reduced by 1 M NaBH<sub>4</sub> in a water bath at 65 °C for 2 h. Reduced N-glycans were desalted and enriched again with GCC-SPE to remove salts from the reduction.

**N-Glycan Preparation.** For simplicity, the compounds were first separated as much as possible using HPLC on a Hypercarb PGC column (Thermo Scientific) (100 mm × 0.5 mm i.d., 5  $\mu$ m particle size) with a Hewlett-Packard series 1100 HPLC system. Fractions were collected in 1 min intervals. A binary solvent system was used comprising of 0.1% formic acid (FA) in 3% ACN in water (v/v) as solvent A and 0.1% FA in 90% ACN in water (v/v) as solvent B. N-Glycans were separated by the gradient: 0–25 min, 0%–15% B; 25.01–50 min, 15–40% B; 50–70 min, 40–100% B at a flow rate of 0.30 mL/min. Fractions were collected in a 70 min run, dried, and reconstituted with nanopure water.

**nanoLC-Chip-Q-TOF MS of N-Glycan.** Each fraction was monitored for N-glycan content using an Agilent 6520 nanoLC-Chip-Q-TOF equipped with an Agilent 1200 series

nano-LC system. The nano-LC system employs a binary solvent system consisting of solvent A, 0.1% formic acid (FA) in 3% ACN in water (v/v), and solvent B, 0.1% FA in 90% ACN in water (v/v). The enrichment column (40 nL PGC) and analytical column (75  $\mu\text{m} \times 43 \text{ mm}$ , 5  $\mu\text{m}$  particle size, PGC) are integrated on a chip, which is placed in the chip-cube interface. The N-glycans were concentrated on the enrichment column for 5 min with 100% solvent A. The instrument was then switched to analysis mode, thereby delivering the N-glycans from the enrichment column to the analytical column for separation. The gradient used was 0% B, 0–2.5 min; 0–16% B, 2.5–20 min; 16–44% B, 20–30 min; 44–100% B, 30–35 min; and 100% B, 35–45 min followed by pure A for 20 min as an equilibration. All MS spectra were acquired in positive mode. The instrument was calibrated prior to use to yield mass accuracies below 5 ppm for MS and 20 ppm for MS/MS.

**Tandem MS and Exoglycosidase Digestion.** Tandem MS analysis was used to guide the exoglycosidase digestions, which in turn were used to elucidate the complete structures. Tandem MS spectra were acquired through collision induced dissociation (CID) by nanoLC-Chip-Q-TOF in the auto MS/MS mode. The applied collision energy depends on the  $m/z$  of each compound according to the formula:

$$V_{\text{collision}} = 1.8 V((m/z)/100 \text{ Da}) - 3.6 V$$

where the slope and intercept were optimized to obtain maximum fragment information.<sup>28</sup>

Buffers for the reaction were prepared by adding glacial acetic acid to 0.1 M ammonium acetate to achieve the desired pH for each enzyme. Reaction time varied from 1 to 24 h at 37 °C depending on the specificity of the enzyme. Reaction conditions for each enzyme are listed in the Supporting Information (Table S2). It is important to point out that the enzyme specificity varied with reaction times. The optimal times were determined in previous studies.<sup>32</sup> The enzymes included  $\alpha(2-3)$ neuraminidase (sialidase),  $\alpha(1-2,3)$ -mannosidase,  $\beta$ -N-acetylglucosaminidase (GlcNAcase),  $\beta(1-4)$ galactosidase, and  $\alpha(1-3,4)$ fucosidase.

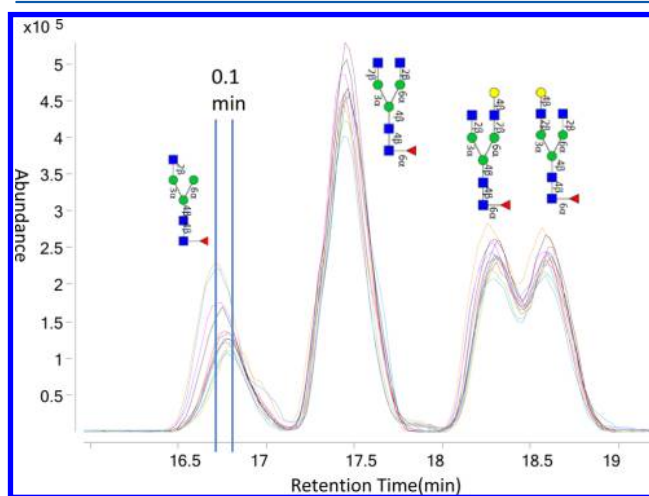
## RESULTS AND DISCUSSION

**Structural Identification of rMab N-Glycans Using a Human Serum Glycoprotein N-Glycan Library.** In a previous study, we published N-glycan structures from a group of the most abundant glycoproteins in serum.<sup>22</sup> Those glycan structures were used to identify some of the structures in the antibodies. The N-glycan library is developed based on released and reduced N-glycans from serum glycoproteins, separated on a PGC column, and analyzed by TOF MS. The linkages were elucidated through a combination of exoglycosidase sequencing, LC, LC-MS, and tandem MS. The comprehensive N-glycan library is composed of complete structures with associated LC retention times, accurate masses, and relative abundances. The mass accuracy of Q-TOF MS used in this study is 5 ppm and the retention time coefficient variance (CV) of the nano-Chip-LC is within 0.6% when run on the same day, which assured the robustness of this approach. The majority of the N-glycans from the eight commercial rMab drugs were characterized by matching the LC retention times and accurate masses with the structures in a previously annotated reference library.<sup>22</sup>

Figure 1 illustrates how the structures were assigned for rMab via LC retention time and accurate mass. Reference

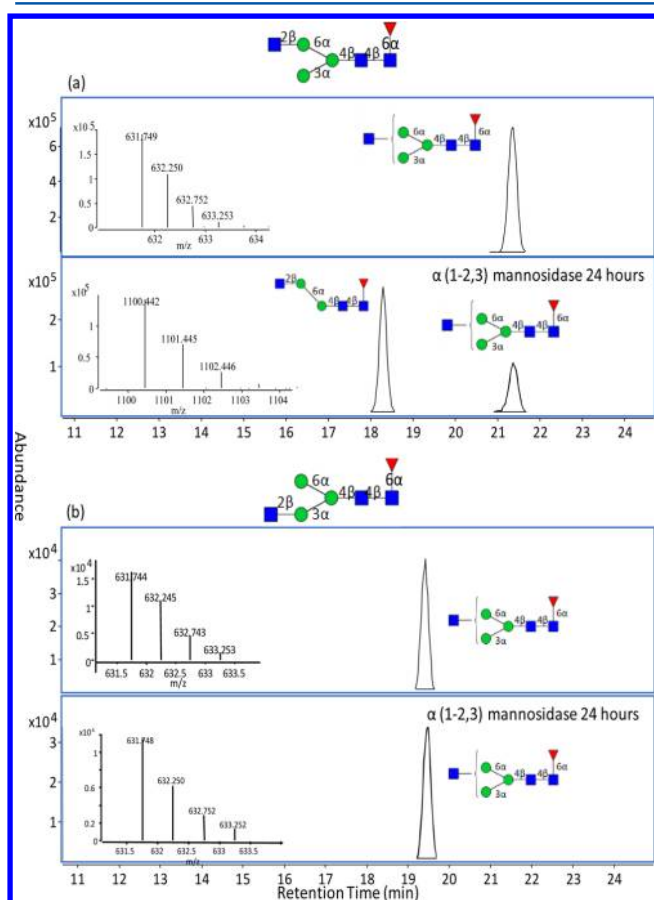
compounds in the library are obtained from serum glycoproteins. Shown in Figure 1 are extracted compound chromatograms (ECC) of several compounds from the reference library and from the antibody drug. In Figure 1a, the upper chromatogram shows the four isomers of N54110 in the reference compound. The nomenclature uses the letter “N” for N-glycan with each digit representing the composition Hex:HexNAc:Fuc:NeuAc:NeuGc. The mass spectrometer does not distinguish between mannose and galactose (Hex) or between N-acetylgalactose and N-acetylglucose amine (HexNAc). However, fucose (Fuc) as well as N-acetylneuraminic acid (NeuAc) and N-glycolylneuraminic acid (NeuGc) are readily determined by mass. NeuGc is not typically found in humans but is found in other mammals.<sup>33</sup> The abundances of NeuGc residues in rMab are low but measurable and are due to the production in CHO/SP 20/NS0 cells.<sup>21</sup> The lower case letters following the numbers represent the order in which the compound was elucidated and roughly follows its relative abundance in serum or its relative abundance in rMab for those not present in serum. The lower chromatogram in Figure 1a shows the ECC of one isomer of N54110 found in Trastuzumab that is aligned with a structure originally found in serum glycoproteins and whose structure is known. Therefore, the compound in rMab is identified as the same structure N54110a in the N-glycan library. The symbolic structure is shown in the inset. Figure 1b demonstrates the identification of N44100a and N44100b in rMab. The complete structures for N44100a and N44100b are given with the isomer “a” having a Gal on the 1-6 arm and isomer “b” with the terminal Gal on the 1-3 arm (structure inset). N44000a and N44000b in Figure 1c and N55110a in Figure 1d were identified in the same way.

In order to further evaluate the robustness of using LC retention times to identify isomers from the N-glycan library, the retention time shift between different runs within 1 day of rMab N-glycans was examined. The retention time repeatability was high with the largest shift in retention time correspond to approximately 0.1 min in a 60 min gradient (Figure 2); however, the shifts are generally much less. This corresponding CV was calculated to be  $\sim 0.6\%$ .



**Figure 2.** Chromatograms of 13 overlaid injections showing the major N-glycans from Panitumumab acquired on nanoLC-Chip-TOF. Injections were performed consecutively. The largest variation in retention time corresponded to 0.10 min.

**Structural Elucidation Using Exoglycosidase Sequencing.** Additional exoglycosidase sequencing was performed to deduce the complete structures of around 30 percent of N-glycans in rMab that were not determined based on the current N-glycan library because they are either absent or in very low abundance. The enzymes are sufficiently specific to linkages for specific digestion periods as previously reported in our laboratory.<sup>29,32</sup> In order to eliminate the interference resulting from isomeric structures during the digestion, off-line HPLC fractionation was performed to isolate the compounds as much as possible. Fractionating the compounds also minimized overlap between enzymatic digestion products. Figure 3



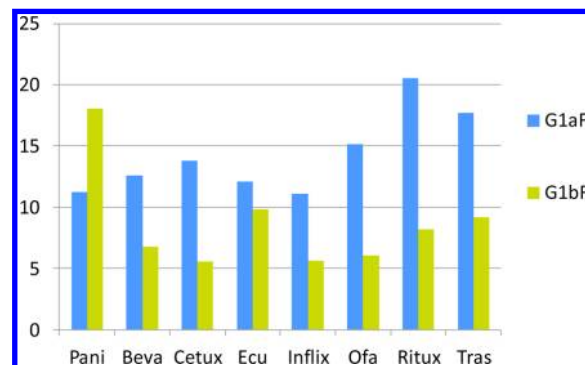
**Figure 3.** Chromatograms (with MS inset) produced during exoglycosidase sequencing of two isomers with composition N33100 (1261.50 Da, neutral mass). (a) The upper chromatogram shows isomer N33100a before digestion. The representation identifies the uncertainty in the structure. The lower panel includes the ECC of the neutral mass 1261.50 and 1099.45 Da. A single mannose was lost after 24-h digestion with  $\alpha(1-2,3)$ mannosidase (MS inset) suggesting an uncapped mannose structure on the 1-3 antenna. The results show that the terminal GlcNAc is on the 1-6 branch. (b) The upper chromatogram shows isomer N33100b before enzyme digestion (MS inset). This compound was analyzed at the same time as the other isomer. There was no loss in signal or new smaller homologue produced when the compound was reacted with  $\alpha(1-2,3)$ mannosidase.

demonstrates the determination of two isomers of N33100 obtained in two separate fractions, using  $\alpha(1-2,3)$ mannosidase. The upper chromatograms in Figure 3a,b show the two isomers before enzyme digestion (MS spectra are the inset). The lower chromatograms show two digestion products with two distinct

masses (MS spectra are the inset). After 24 h of  $\alpha(1-2,3)$ mannosidase digestion, the isomer in Figure 3a lost one mannose while the isomer in Figure 3b remained the same mass thereby showing that the first has the terminal mannose on the 1-6 arm while the second has the terminal mannose on the 1-3 arm. The same method employing enzyme digestion monitored by LC-MS were applied to deduce the other structures in rMabs. The retention times of all the new N-glycans were noted and used for future structural identification in other rMabs.

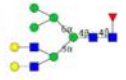

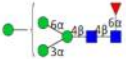
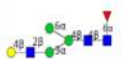
**N-Glycan Library for rMab.** The completed N-glycan library for rMab includes all the N-glycans from the commercial rMab drugs detected by nanoLC-Q-TOF MS. Relative abundances were acquired for each N-glycan based on the integration of the ion abundances associated with each peak and normalized to the total ion counts of all the N-glycans of each rMab. The rMab N-glycan library contains more than 70 entries spanning 3 orders of magnitude in abundances. There are 25 fully elucidated structures, with the remainder being partially elucidated structures. The latter group is of low abundances and could not be isolated in sufficient amounts for complete elucidation. The fraction of fully elucidated structures represents more than 80–90% of the total abundances for each rMab. This group represents a significantly more comprehensive one than the standard G0F, G1F, and G2F.<sup>11,12,20,34–36</sup> N-Glycans with NeuGc residues were also included in the library, and however their abundances were usually less than 5% of the most abundant species.

Table S1 in the Supporting Information presents the comprehensive library, with systematic names, accurate masses, symbolic structures annotated with linkage information, and associated rMab drugs. The top 10 N-glycans were found in all rMab drugs included in this study. Most isomers were well separated and characterized. The most abundant compounds generally contained complete structures and are shown with all linkage information. The common structures referred to as G1F, G0F-GlcNAc, G1 correspond to the respective pairs N44100a and N44100b, N33100a and N33100b, and N44000a and N44000b.<sup>11,12,20,21,35–37</sup> The extracted chromatograms provide relative quantitative information. For example, the two isomers, N44100a and N44100b have significantly different abundances with N44100a (Gal on the 1-6 antenna) almost twice as abundant as N44100b (Gal on the 1-3 antenna) in Trastuzumab, Bevacizumab, Rituximab, Cetuximab, Infliximab, Ofatumumab, and Eculizumab. In contrast, N44100b is twice as abundant as N44100a in Panitumumab (Figure 4). The most



**Figure 4.** Relative abundances of N44100a (G1aF) and N44100b (G1bF) for each rMab.

Table 1. Top Five Most Abundant N-Glycans in Each rMab with Nonstandard N-Glycans Shown As Symbolic Structures<sup>a</sup>

Abundance order	Beva	Cetux	Ecu	Inflix	Ofa	Pani	Ritux	Tras
1	N34100a	N34100a	N34100a	N34100a	N34100a	N34100a	N34100a	N34100a
2	N44100a	N44100a	N44100a	N44100a	N44100a	N44100b	N44100a	N44100a
3	N44100b		N44100b	N44100b	N44100b	N44100a	N44100b	N44100b
4	N34000a	N44100b	N54100a		N54100a	N54100a	N54100a	N34000a
5	N33100b	N54100a	N33100b	N33100b			N34000a	N54100a

<sup>a</sup>The remaining structures are listed in Table S1 in the Supporting Information.

abundant N-glycans generally corresponded to G2F, G1F, G2F, and Man5 as stated in previous reports.<sup>12,20,21,34–38</sup> However, we find that other N-glycans may be as abundant (Table 1). For example, N74100a is even more abundant than N44100b (G1bF) and N54100a (G2F) in Cetuximab. Similar observations are found with Infliximab, Ofatumumab, and Panitumumab, where noncommon structures were more abundant than those commonly monitored. Cetuximab and Infliximab (produced in Sp2/0 cell lines) and Ofatumumab (produced in NS0 cell lines) are found to have a higher percent of NeuGc-containing N-glycans (6.81%, 6.51%, and 5.16%, respectively) compared to Trastuzumab, Bevacizumab, Rituximab, and Panitumumab (0%, 4.15%, 3.67%, and 0%, respectively), which are produced in CHO cell lines. This finding is consistent with a previous report that CHO cell lines may yield lower abundances of NeuGc-containing N-glycans.<sup>16</sup>

## CONCLUSION

We constructed an N-glycan library containing complete structures for rapid and high-throughput glycan analysis of rMab. This library provides a reference for the rapid identification of glycans in rMab and possibly other biologics that are produced with the same cell lines. The reproducible LC retention times and accurate masses are useful markers for identifying structures. It no longer requires further time-consuming N-glycan isolation and extensive exoglycosidase sequencing.<sup>22,29,39</sup> The tandem mass spectra for each N-glycan were also recorded in the library but were seldom used for further identification (Figure S1 in the Supporting Information). For the utility of the N-glycan Library, a 0.1 min retention time window and 20 ppm mass accuracy window are suggested as the minimum criteria for identification.<sup>22,29,39</sup>

## ASSOCIATED CONTENT

### Supporting Information

Complete table of rMab N-glycan library and figures as noted in text. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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### Notes

The authors declare no competing financial interest.

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