A middle-down NMR protocol for therapeutic mAb glycan profiling

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Abstract:

Recombinant monoclonal antibody (mAb) use is growing across various therapeutic areas. During production, host cell enzymes glycosylate target mAb proteins. Monitoring glycan distribution is essential for ensuring mAb product quality, manufacturing consistency, and assessing biosimilarity between manufacturers. A middle-down NMR method has been developed to profile mAb glycan distributions, where glycans remain covalently attached to the mAb's fragment crystallizable (Fc) domain. This technique uses the immunoglobulin-degrading enzyme IdeS from *Streptococcus pyogenes* to cleave the Fc domain. The purified Fc domain is then denatured and dissolved in urea, and a high-resolution 2D ^{1}H-^{13}C HSQC NMR spectrum is collected. The resulting anomeric peak distribution reveals major and minor glycan species, including the trimannosyl core, high-mannose structures, and branch-specific galactosylation patterns. Compared to the traditional glycan mapping method, which involves enzymatic cleavage and liquid chromatography (LC) separation, the middle-down NMR approach offers a non-invasive, comprehensive analysis that preserves glycan integrity and ensures full monosaccharide coverage. The method takes 3-4 days to complete, with approximately 4-5 hours of hands-on time. It can be easily implemented in regulated environments for both development and quality control workflows, especially where high-resolution glycan profiling is crucial. Basic biochemistry and 2D NMR analysis expertise are required to perform this method.

Key points

- The middle-down NMR protocol is well-suited for profiling the glycan distribution of therapeutic monoclonal antibodies (mAbs), preserving the covalent linkage between the glycan and the mAb.
- The chemical shift values of Fc-associated glycans in ^{1}H-^{13}C HSQC spectra remain consistent,

enabling reliable spectral interpretation.

- Spectral libraries acquired in previous years can be reused for comparative analysis with new mAb product batches, supporting long-term analytical consistency.
- This method is applicable in mAb drug development and biosimilar evaluation, aiding in candidate screening, quality assurance, and detailed characterization.

Introduction

Monoclonal antibody (mAb) based protein drugs are continuously being developed to target a range of inflammatory diseases and cancers given their specificity and efficacy.[1, 2] A multi-domain mAb protein is composed of a 100 kDa dual-domain antigen binding fragment F(ab')2 and a 50 kDa crystallizable fragment (Fc). The production of recombinant mAb drugs takes place in mammalian cells. The critical quality profile defining an mAb comprises the protein sequence, the higher order structure, and the protein post-translation modifications (PTMs).[3, 4] One major PTM of mAbs potentially impacting therapeutic efficacy is N-glycosylation on the asparagine-297 sidechain of the Fc domain, which introduces chemical heterogeneity in the mAb product and modulates mAb function through interaction with cell receptors.[5, 6] The heterogenous glycan structure is known to affect mAb effector function, drug efficacy, and clearance.[6] For example, complex afucosylation has been observed to increase antibody-dependent cellular cytotoxicity (ADCC);[7, 8] high mannose was found to be associated with fast plasma clearance;[9] and galactosylation could affect complement-dependent cytotoxicity (CDC).[10] Accurate and complete measurement of glycan distributions is a necessary characterization during mAb drug development and quality assurance.[11] NMR based analytical measurements are non-invasive and intrinsically mass balanced because the physical basis of the technique is inherently quantitative. Thus, following a urea denaturation approach

developed by Schubert et al,[4] a middle-down NMR workflow was developed, in which the Fc domain was enzymatically cleaved from the mAb, separated, and denatured for NMR measurement.[4, 12, 13] The glycans remain covalently linked to asparagine on the Fc domain and with protein denaturation, the dynamic motion of the glycans increase which leads to improved sensitivity such that the cross peak signals can be measured without isotopic enrichment using high-resolution 2D ^{1}H-^{13}C HSQC spectra at ^{13}C natural abundance (*ca.* 1.1%). The ^{1}H and ^{13}C chemical shifts of anomeric peaks resolve different monosaccharide types, configurations, and the peak intensities which can be used to determine glycan abundance.[14, 15] The middle-down NMR method was demonstrated to differentiate therapeutic mAbs of varied glycan distribution,[16] with the capability to identify and quantify major[12] and minor glycans.[13] As the NMR measurements are inherently quantitative and reproducible if run under the similar conditions, library spectra of mAb glycan profile[17] acquired over time are relevant comparators for product stability across manufacturing sites and batches or for biosimilar products manufactured differently.[18, 19]

- **Development of the protocol**: The middle-down procedure follows the "middle-down" mass spectrometry (MS) approach of measuring only the Fc domain of mAbs.[20, 21] A highly specific immunoglobulin-degrading enzyme from *streptococcus pyogenes* (IdeS) was chosen to digest the Fc domain from the mAb at a single cleavage site below the hinge region.[12, 22] The lower PTM complexity of the Fc domain allows the glycan distribution to be exclusively studied using MS. However, unlike MS, the 2D ^{1}H-^{13}C HSQC NMR at natural abundance requires a protein quantity in milligrams (mg), which necessitates the separation and enrichment of the Fc domain. A non-affinity based size-exclusion fast protein liquid chromatography (SEC-FPLC) approach was chosen to separate the Fc domain after IdeS digestion and assure complete digestion. The

fractions corresponding to the Fc domain were pooled and dialyzed against pure water to remove buffer salt from the SE running buffer. The salt free Fc sample was lyophilized and redissolved in deuterated urea solvent for NMR measurement.[4] During development, it was found that a Fc protein quantity of 40 mg was required for each NMR sample preparation.

- **Applications of the method**: For therapeutic protein development, ensuring the quality of glycan distributions such as fucosylation, mannosylation, and galactosylation, is critical. Comprehensive glycan profiling requires analytical methods that offer complete coverage and reproducibility.[19, 23] The mass-balanced, non-invasive, and reproducible NMR protocol described here is well-suited for monitoring glycan profiles on proteins.[24] While the U.S. Food and Drug Administration (FDA)[25] and the European Medicines Agency (EMA)[26] do not explicitly reference NMR in their biosimilar guidelines, both agencies emphasize the application of advanced, state-of-the-art analytical technologies to thoroughly characterize biological products. Uniquely the chemical shift measurement from the middle-down NMR approach enables the validation of glycan structure of anomeric configuration and linkage, not readily available from other methods. Thus, NMR, with its ability to resolve chemical and higher-order structural features, aligns well with these regulatory expectations and offers complementary insights into the manufacture control and biosimilarity assessment of complex biologics such as oligosaccharides[27] and mAbs.[28, 29]

Moreover, this technique could be leveraged as a diagnostic tool to identify biomarkers, such as the α-Gal epitope in mAbs[30] and inflammatory glycopeptides in human serum samples.[31, 32] Beyond glycosylation analysis, middle-down NMR has the potential to detect chemical modifications such as phosphorylation, acetylation, and oxidation in proteins through chemical

shift changes.[33-36] In summary, the middle-down NMR supports broader glycoprotein structural and functional studies, reinforcing its value as a research, manufacturing control, and analytical platform.[36, 37]

- **Comparison with other methods**: The standard glycan mapping analytical procedure includes glycan cleavage from the protein, labeling with a fluorescent tags, and separation by LC for quantitation using fluorescence detection.[17, 38] Other intact and middle-down MS approaches use direct infusion MS for measuring glycan distribution, which quantifies major glycan pairs based on MS signal intensity.[39] Recently, a multi-attribute method (MAM) was developed where glycol-peptides are generated from mAb enzymatic digestion and quantified for glycan distribution analysis using high-resolution MS signals. However, with the MAM approach there is potential variability originating from the digestion procedure.[40] While the MS method is sensitive, resolving isobaric species by MS is challenging, e.g., the same MW species which can result from a single terminal galactosylation at the 1-3 and 1-6 branches. A newer glycan mapping method has been shown combining LC separation followed by tandem MS to identify and quantify co-eluting glycans.[41] The middle-down NMR protocol offers an orthogonal method for glycan profiling at the protein domain specific level with glycans remaining covalently linked to proteins, which represents a milder treatment of glycans compared to all other glycan measurement methods. Besides experimental procedure differences, the data analysis is also different between all the available methods and NMR. Glycans are shown in the 2D NMR spectra as anomeric peaks of each monosaccharide, which could belong to several glycans with a similar local structure, e.g., the same trimannosyl core structure is conserved in different N-glycans. As a result, NMR is considered a profile method although converting to a grouped glycan abundance could be performed.[13]

- **Experimental design**: The NMR signal depends on molecular rotational dynamics with small molecules possessing fast motion which is more advantageous for lineshape and the associated sensitivity of the measurements. Glycans are oligosaccharides covalently linked to large proteins and are intrinsically less dynamic because of tethering, thus are not suitable for NMR data collection. Protein denaturation in urea solutions unfolds protein higher order structure into less folded random coil type structures for the polypeptide chains, therefore, the overall molecular dynamic motions including the proteoglycans increases, becoming suitable for NMR data collection.[4] However, to further increase the solubility of glycans, the mAb protein needs to be enriched with the protein component that contains the glycans, i.e., the Fc domain, which necessitates the middle-down procedure. The middle-down approach starts from IdeS enzymatic digestion of full length mAb (Figure 1). To ensure mass balance, a non-affinity-based SEC column is used to monitor digestion completion and assure complete recovery of Fc domain fractions. Overnight digestion is sufficient and SEC-FPLC with a Superdex-75 column is ideal for resolving peaks of 150-kDa mAb, 100-kDa F(ab')2 and 50-kDa Fc. NMR samples, if directly concentrated from SEC fractions, will be in high salt, not suitable for NMR probe. Thus, a dialysis step is necessary to remove salt from the Fc samples. After fraction collection and lyophilization, the Fc powder can be directly dissolved in 7.8 M urea with DTT for NMR data collection.

Materials

Reagents

- IdeS Protease (Promega, Ref. V751A)
- PBS buffer, Phosphate Buffered Saline 10X molecular biology grade (Mediatech, Inc., REF No. 46-013-CM)

- H_2O, water, Nuclease-free, molecular biology grade, ultrapure (Thermo Scientific, cat. No. J71786-AE)
- D_2O, deuterium oxide, D, 99.9% (Cambridge Isotope Laboratories, Inc., cat. No. DLM-4-100)
- Urea-d_4, D4, 98% (Cambridge Isotope Laboratories, Inc., cat. No. DLM-1269-25)
- DTT-d_{10}, DL-1,4-Dithiothreitol, D_{10}, 98% (cat. No. DLM-2622-1)
- DSS-d_6, 3-(Trimethylsilyl)-1-propanesulfonic acid-d_6 sodium salt (Sigma Aldrich, cat. No. 284664-85-3)
- H_2O, water, for FPLC
- Dry ice

Drug product

- Humira (Adalimumab, 40 mg/0.8 mL, NDC 0074-3799-02)

Equipment

- FPLC system (AKTA pure chromatography system, Cytiva)
- FPLC-SEC column (Hiload 16/600 Superdex-75 prep grade, Cytiva)
- Water purification system (ELGA Ultrafilter Endoguard 5000 MWCO, Purelab Ultra)
- Corning Falcon centrifuge tubes, 15 mL and 50 mL
- Microcentrifuge tubes, 0.6 mL and 1.5 mL
- NALGENE 0.2 μm PES Rapid-Flow bottle top filter (Thermo Scientific, cat. No. 595-4520)
- NALGENE receiver bottle, 1 L (Thermo scientific, cat. No. 455-1000)
- Dialysis tubing (Spectra/Por® Dialysis Membrane, 8-10 kDa MWCO)
- Beaker (Nalgene, cat. No. 1201-4000, 4 L)
- Centrifuge (Thermo Scientific, Sorvall Legend XTR)
- Freeze Dryer (Labconco, 2.5 Liter -84C Benchtop)

- NMR (Bruker, 500 MHz)
- NMR tube (Shigemi tube, OD: 5 mm, Wilmad-LabGlass)
- Topspin (Bruker)
- Mnova (Mestrelab, Version 15.0.1)

Reagent setup

0.2X PBS buffer for FPLC

PBS buffer is prepared from a 10X concentrated PBS solution. 20 mL of 10X concentrated PBS solution is mixed with 980 mL water from the water purification system. The 0.2X buffer solution is filtered through a 0.2-micron filtration system and left on a vacuum to degas for 30 min.

7.8 M urea-d_4 in D_2O

5 g of urea-d_4 is weighted out in a 15 mL Falcon tube then D_2O is slowly added (containing 20 μg/mL DSS) until all the urea-d4 dissolved; filling the Falcon tube to the 10 mL mark. About 4-5 mL D_2O is needed to make a 10-mL stock solution. The solution is stored at room temperature.

200 mM DTT-d_{10} in D_2O

38.5 mg of DTT-d_{10} is dissolved in 1.17 mL of D_2O (contained 1.2 μg/mL DSS) in a 1.5 mL microcentrifuge tube, then aliquoted into 0.6 mL microcentrifuge tubes, with 0.055 mL DTT-d_{10} solution in each aliquot. The aliquots are stored in -20 °C freezer.

Equipment setup

- **Limitations**: Glycans with sialic acids at amounts at 1% or below of the parent peak are not detected due to sensitivity issues at these amounts.

Procedure

- **Fragmentation**

1. A total of 3.8 mL Humira® drug product (total of 190 mg adalimumab) is transferred to a 15 mL Falcon tube. One vial of 5000 units of IdeS protease is dissolved with 0.1 mL H_2O, then transferred to the same Falcon tube containing adalimumab. The Falcon tube is centrifuged for 1-minute at 1000 rpm to bring all the solution to the bottom of the tube. The tube was left at room temperature for 16 h for the reaction to complete before transferring to a 4 °C refrigerator for storage.

- **Purification**

2. To separate the Fc domain, the IdeS digested adalimumab solution is subjected to SEC-FPLC equipped with a Hiload 16/600 Superdex-75 pg column. For each run, 1 mL of digestion solution is injected, flow rate is 1 mL/min, and the running buffer is 0.2x PBS buffer. A total of 4 runs are performed. The eluted Fc fractions (FPLC chromatogram in Figure 1) are combined to give a total of 45 mL solution.
3. The Fc solution is transferred to an 8-10 kDa MWCO dialysis tubing (18 cm in length), which is then dialyzed against ultra purified water in a 4 L beaker for 16 h at 4 °C .
4. The dialyzed sample (~45 mL) is collected in a 50 mL Falcon tube and frozen on dry ice for 1 h. The frozen Fc sample is lyophilized on a freeze dryer until dry. The quantity of Fc domain should be 63 mg assuming no loss.

- **Denaturation**

5. An aliquot of 200 mM DTT in D_2O (0.055 mL) is thawed then 0.05 mL of DTT is mixed with 0.45 mL of 7.8 M urea in D_2O in a 1.5 mL microcentrifuge tube. A 0.4 mL aliquot of the urea/DTT solution is then transferred to the Falcon tube containing the freeze-dried Fc powder to give ~160 mg/mL sample concentration. The Falcon tube is gently swirled for dissolution, then is centrifuged to bring all the solution down to the bottom of the Falcon

tube. An 0.25 mL aliquot of the Fc in urea solution is directly transferred to a Shigemi NMR tube.

- **NMR solvent sample**

An optional NMR blank solvent sample can be prepared using 7.8 M urea containing DSS in D_2O for chemical shift reference.

- **NMR:**

6. 1D ^{1}H NMR: All NMR spectra are collected at an experimental temperature of 30 °C using a Bruker 500 MHz spectrometer equipped with a cryogenic TCI probe. The 1D ^{1}H qNMR spectrum is collected using a *zgpr* modified pulse program *qzgpr.kc*, which separates the water presaturation period from the recycle delay.[15] The spectrum is referenced to the methyl peak of DSS at 0 ppm. The ^{1}H carrier is placed on the HDO resonance at 4.70 ppm. The spectral width is 15 ppm, and a total of 7812 complex points are collected. The acquisition time, recycle delay and presaturation period are 1 s, 3 s and 3 s, respectively. The number of scans is 64. The free induction decay (FID) is apodized with a 0.3 Hz exponential decay function, phase corrected and zero filled to a spectral size of 32k points before Fourier transform (FT). A 3rd order polynomial function is applied for baseline correction. All the 1D NMR spectra are processed and analyzed using MestReNova 14.2 software (Mestrelab Research S.L.).

7. 2D ^{1}H-^{13}C NMR: The two 2D ^{1}H-^{13}C HSQC NMR spectra are collected for the Fc sample following the published literature,[15] in which a modified sensitivity enhanced gradient HSQC pulse sequence *hsqcetgpsi2.kc* is applied.[13] The spectral width for the ^{1}H dimension is 12 ppm with the carrier frequency centered at 4.70 ppm, whereas the spectra width of the ^{13}C dimension is 100 ppm with the carrier frequency centered at 102 ppm for HSQC.

The complex points of 640 and 3018 are acquired for the ^{1}H and ^{13}C dimensions, respectively. The resulting acquisition times for ^{1}H is 100 ms and ^{13}C is 120 ms. The ^{13}C decoupling sequence is GARP with a radio frequency field strength of 1.25 kHz. The coupling constant $^{1}J_{HC}$ is set to 180 Hz as a compromise between the efficiency of Insensitive Nuclei Enhanced by Polarization Transfer (INEPT) transfer and T_2 signal loss. The recycle delay is 3 s. The number of scans is 16 and the total experimental time is 42.5 h. The FIDs of both ^{1}H and ^{13}C dimensions are apodized with a 90° shifted sine-square function, followed by scaling the first point to 0.5, and zero filling to 4k real data points. Zero-order phase correction is applied to the ^{1}H dimension. The ^{13}C chemical shift is indirectly referenced following an established Ξ ratio of 0.251449530 of DSS.[42]

Timing:

Fragmentation: Overnight (16 h) at room temperature is sufficient. Hands on time is 10 min.

Purification:

- FPLC: Each FPLC purification requires approximately 2.5 h, however the total FPLC purification time varies depending on the number of injections. For 190 mg adalimumab, the total purification time is 10 h (4 runs in total). Hands on time is 10 min per injection.
- Dialysis: Dialysis can be done at room temperature for 4 h or at 4 ^{o}C overnight (16 h). For this protocol, dialysis is done at 4 ^{o}C overnight. Hands on time is 30 min for setting up dialysis.
- Freeze dry: Depending on the amount of solvent, the freeze dry time varies. For this protocol, the sample is put on a freeze drier for 4 days (over the weekend). Hands on time is 60 min for setting up the freeze dryer.

Denaturing: The Fc protein needs to be dissolved in urea for denaturation. Hands on time is 30 min including NMR sample preparation.

NMR: Each ^{1}H NMR takes around 10 mins. Each 2D ^{1}H-^{13}C HSQC NMR takes 42.5 h. Hands on time for NMR experiment set up is approximately 30 min.

Troubleshooting:

Step	Problem	Possible reason	Solution
2	Area% for Fab and Fc region is not around 2:1	Fragmentation may not be complete	Allow fragmentation at room temperature for another day.
2	Fab and Fc have large overlap due to wide peak distribution	Not enough salt concentration	Increase the PBS buffer concentration from 0.2x to 0.5x.
5	Fc does not dissolve in urea/DTT solution	DTT may be bad	Make new DTT solution. Excess DTT solution should be stored at -20 ^{o}C or below in aliquots and avoid repeating thawing procedure.
6	DSS signal is not sharp	DSS might interact with protein	Take a ^{1}H NMR of 7.8 M urea-d_4 in D_2O containing DSS for external reference.
7	Extra peak at $^{1}H/^{13}C$ chemical shift of 4.45/104.7 ppm	Cellulose molecule	Change the vendor of dialysis tubing.

Anticipated results:

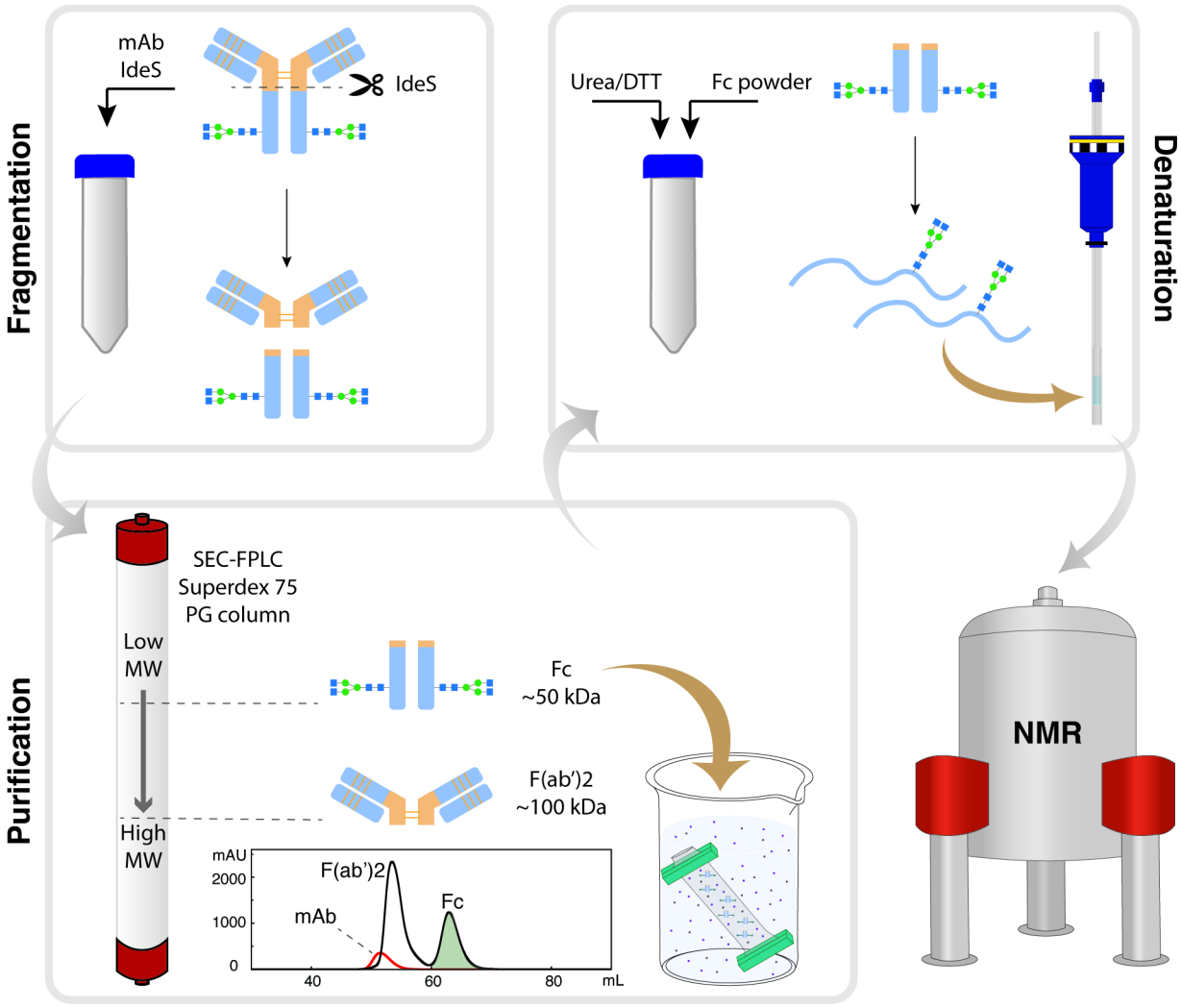
The 1D ^{1}H NMR spectrum of NMR solvent shows signals of solvent urea and the spiked DTT (Figure 2A), which can be used to externally reference the chemical shift 1D and 2D spectra of adalimumab-Fc (Figure 2B and 3). The glycan signal in the 1D ^{1}H NMR spectrum of adalimumab-Fc is buried in the protein signal, thus is not informative on glycan structure. The 2D ^{1}H-^{13}C HSQC spectra show well-isolated cross peaks in the anomeric region and their ^{13}C chemical shifts resolve each monosaccharide (Figure 3). The peaks identified with s/n above 6.5 are GlcNAc1-2, Fucose, Man3-4, GlcNAc5, ManA-C, and Gal6 (Figure 3). An unknown peak with s/n above 9.5 is located at 4.96 ppm for ^{1}H and 100.42 ppm for ^{13}C. The GlcNAc1 has a ^{13}C chemical shift of 81.13 ppm which is up field compared with all other anomeric peaks due to its covalent linkage of an Asn sidechain (N-

linked). The rest of the anomeric signals range from 99 to 106 ppm. The peak intensity directly reflects monosaccharide abundance and dynamics. A simple peak profile calculated using Eq. 1, where each peak height is normalized against Man3, illustrates the change in glycan distribution if any. The current profile (Figure 4) shows consistency to the previously published adalimumab glycan peak profile.[13] Additionally, peak intensity can be used to calculate minor glycan quantity like high-mannose using Eq.2. The (M4-M9) % for adalimumab is 7.05%, within the range of the previous value of 7+/- 1%.[13]

$$\boldsymbol{Profile\ ratio} = \frac{\boldsymbol{Monosaccharide}}{\boldsymbol{Man3}} \qquad \textbf{Eq. 1}$$

$$(\boldsymbol{M4} - \boldsymbol{M9})\% = \frac{\boldsymbol{Man4(6,HM)}}{\boldsymbol{Man4(6)} + \boldsymbol{Man4(6,T)} + \boldsymbol{Man4(6,HM)}} \times \mathbf{100} \qquad \textbf{Eq. 2}$$

In summary, the presented middle-down NMR protocol serves as an orthogonal method to measure glycan structure, species and quantity. The NMR approach retains the chemical bond between glycan and protein; therefore, it has a complete coverage of the glycan profile. The method could also be applied to other complex polysaccharide drugs for quality assurance in addition to research applications.



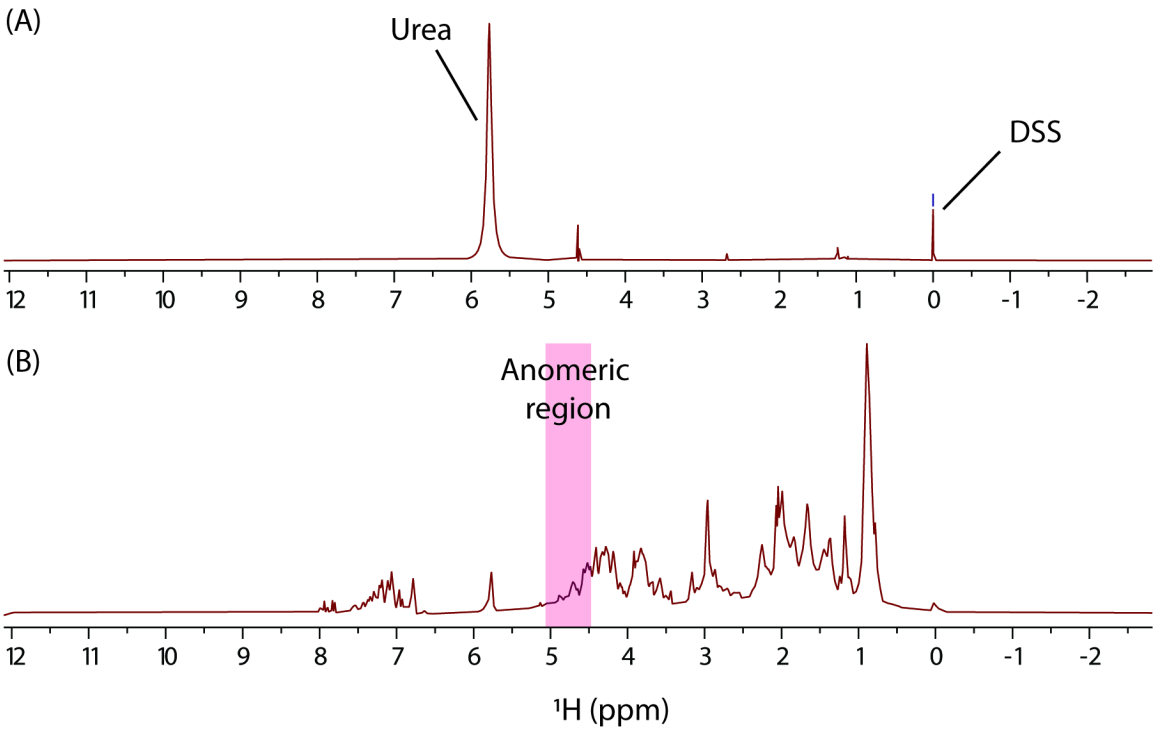
311

312 Figure 1. The middle-down NMR workflow for mAb glycan profile. Three main procedures are

313 fragmentation, purification, and denaturation. Shigemi NMR tube is used to account for the small

314 sample volume.

315



316

317 Figure 2. 1D ^{1}H NMR spectra of (A) 7.8 M urea-d4 in D_2O containing 20 μg/mL DSS and (B)

318 adalimumab-Fc in 7.8 M urea-d4 in D_2O containing 20 μg/mL DSS with 20 mM DTT.

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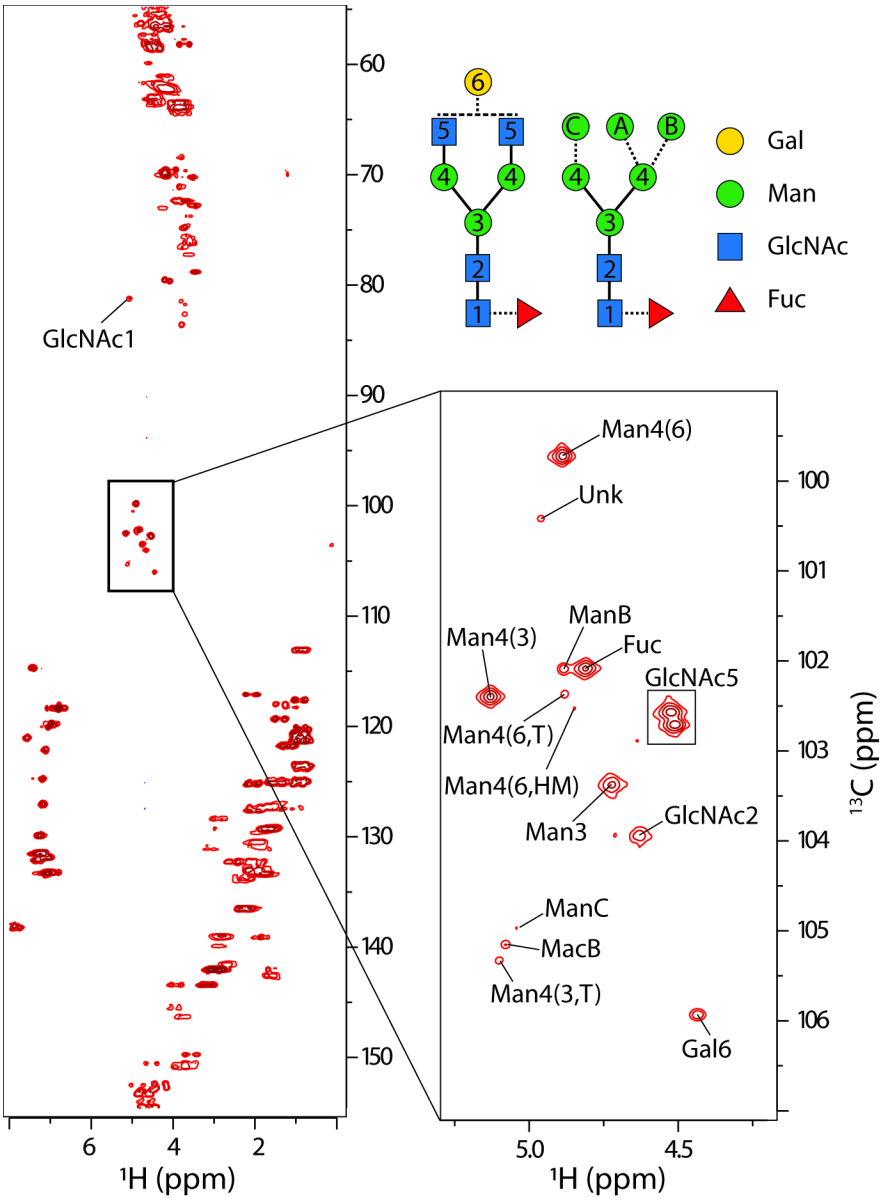

Figure 3. Full (left) and the expanded anomeric region (right) of 2D ^{1}H-^{13}C HSQC spectrum of adalimumab-Fc sample. Major complex and high-mannose glycan structures are drawn in cartoon forms. Abbreviations are Gal for galactose, Man for mannose, GlcNAc for N-acetyl glucosamine and Fuc for fucose.

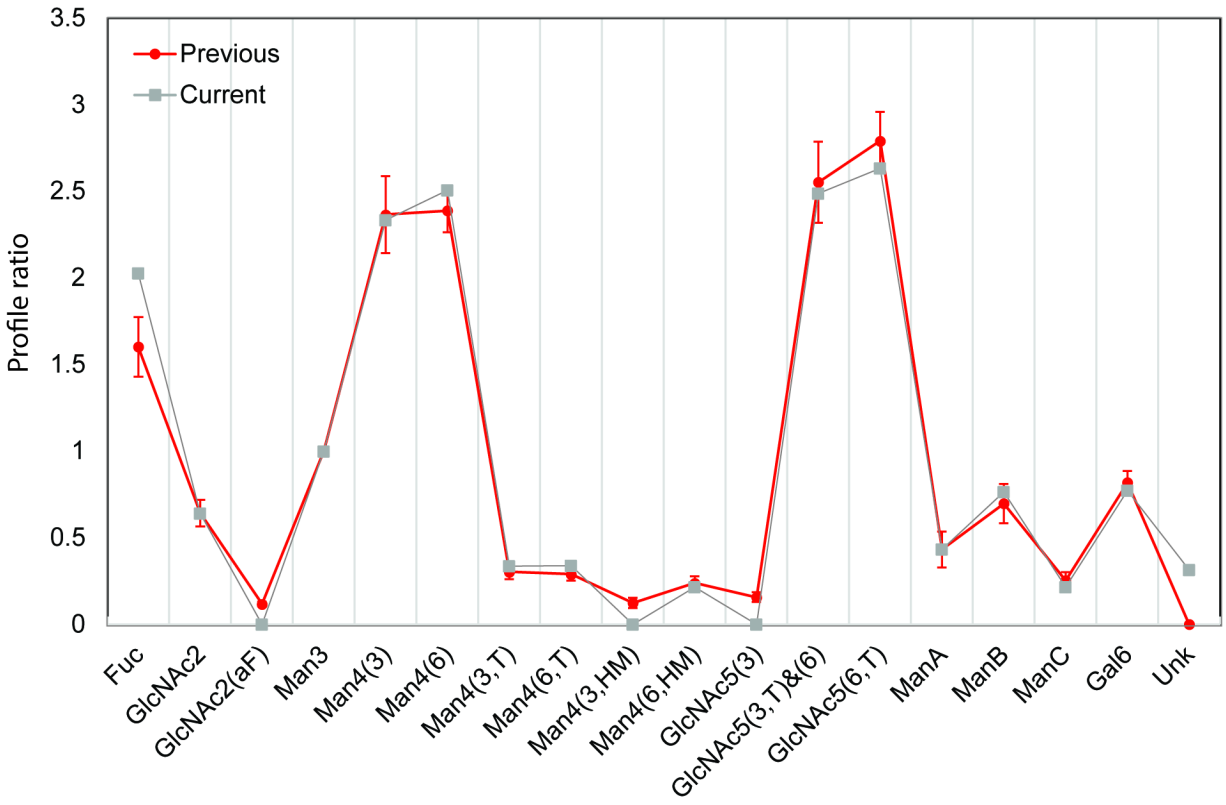

Figure 4. Anomeric peak profile using the current spectrum (gray squares) and the previous spectra[13] (red circles).

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