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LC/MS-based Intact IgG and Released Glycan Analysis for Bioprocessing Applications

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Robust plate based antibody glycan analysis platforms are urgently needed for biopharmaceutical development and manufacturing as well as for clinical biomarker research. A 96-well plate based workflow has been developed to analyze both intact IgG antibodies and released N-glycans using an Orbitrap Fusion Mass Spectrometer and an LC/MS method on the Waters UNIFI platform. Here, such a workflow including protein A purification, PNGaseF digestion, 2-AB labeling, and SPE clean-up is described. The measured IgG glycan profile is consistent with that obtained from non-plate based method and commercial kit and has the advantage of less hands-on time. Also the application of the workflow in cell culture monitoring and clonal selection work is demonstrated. Apart from checking the major glycan structure changes among clones, post translational modifications (PTMs) such as C-terminal lysine residue clipping and N-terminal pyroglutamic acid formation can also be deduced from the workflow.

1. Introduction

Product characterization of therapeutic antibodies is crucial for drug development and manufacturing. Various post translational modifications (PTMs) including glycosylation, pyroglutamic acid formation, and lysine clipping can impact the efficacy and safety profile of the drug[1] hence both the World Health Organization (WHO) guidelines and International Council of Harmonization (ICH) Q6B mandate adequate characterization of the PTMs of therapeutic proteins.[2] In particular, glycan profiling is one of the Critical Quality Attributes (CQAs) of Quality by Design (QbD) framework[3] and many strategies have been proposed and used to fine-tune the glycan structures during cell culturing.[4] Besides monitoring product quality, glycan profiling can also help in clinical research to achieve better diagnosis and uncover mechanisms of diseases such as diabetes and Inflammatory Bowel Disease (IBD).[5,6] In general, glycosylation alteration in tissues from the patients may serve as a biomarker to help in early diagnosis. The underlying molecular basis for such observed phenotypic change in glycosylation may link to the disease mechanism and hence can provide further hypothesis to test.

2-AB labeling of the enzymatically released glycans followed by HILIC separation is a widely adopted strategy helped by GlycoBase for glycan structure identification based on Glucose Unit (GU) computation.[7,8] Workflows using this approach were reported with variation in separation method, clean-up step, and automation platform.[9–11] Apart from 2-AB labeling, other fluorescent reagents such as 2-aminobenzoic acid (2AA),[12] 1-aminopyrene-3,6,8-trisulfonic acid (APTS),[13] Instant AB,[14] RapiFluor-MS,[15] and carbamate[16] have all been used in the detection and quantification of either released glycans or digested glycopeptides[17,18] by HILIC Liquid Chromatography, Capillary Electrophoresis, or MALDI-TOF.[19,20] Among them, MALDI-TOF detection of glycopeptide offers a quick semi-quantitative glycan profiling solution with less sample preparation steps needed but it lacks the ability to differentiate isobaric structures.

Top-down analysis of intact IgG has the advantage of minimal sample preparation. High resolution mass analyzers can be used to monitor glycosylation on the intact proteins.[21,22] Quantification of IgG glycosylation using LCMS based methods was compared to the LC method to demonstrate its usefulness in N-glycan analysis.[23] Intact IgG analysis is increasingly used in bioprocess monitoring[24,25] and fast biosimilar characterization[26] and provides information about the observed pairing of glycan on IgG.[27]

In this work, we described the development of a semi automated plate-based workflow for glyco-analytics on both intact and released glycan levels. This optimized workflow was then used to monitor glycan changes in a bioreactor at various

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time points and obtain the glycan profile in clonal selection work for biosimilar development.

2. Experimental Section

2.1. Consumables

MultiScreen Filter Plates with Ultracel-10 Membrane (Millipore) were used to separate the released glycan from the protein after PNGaseF (New England BioLabs) treatment. A 2 AB labeling of free glycan was performed with sodium cyanoborohydride from Sigma–Aldrich and anthranilamide from ACROS Organics while the reaction solvents such as dimethyl sulfoxide, acetic acid, and acetonitrile were all purchased from Merck. The labeling reaction was performed on a 96 well Eppendorf PCR plate (AIT Biotech). The excess dye was removed on a Hypersep® DIOL SPE Plate from Thermo Scientific.

2.2. Protein A Purification for Supernatant

A 96-wells IgG affinity purification plate (Thermo Scientific) was preconditioned by washing with 400 µl of 1xPBS. Supernatant was transferred into well and reloaded into the wells for three times. The resins were then washed with 6×400 µl of water. Elution buffer of 3×200 µl 0.1% formic acid (Merck) per well was used with vacuum filtration. 20 µl of neutralization buffer (1M Tris buffer pH 8.0, 1st Base) in each well was used to adjust pH.

2.3. Glycan Release and Labeling

The purified IgG was transferred into a 96-well ultrafiltration plate (MultiScreen Filter Plates with Ultracel-10 Membrane, Millipore) and the solvent was removed by centrifugation ($2000 \times g$, 40 min). For each well, water (178 µl well^{-1}), Reaction Buffer (0.5 M sodium phosphate pH 7.5, 20 µl well^{-1}, New England BioLabs) and recombinant PNGaseF (2 µl well^{-1}, 1000 U, New England BioLabs) were added. The plate was sealed and incubated at 37 °C on AssayMap Bravo platform (Agilent) with agitation at 700 rpm for 2 hr. Released glycans were recovered from the plate by centrifugation ($2000 \times g$, 40 min) and transferred into a 96-well PCR plate. A total of 20 µl of labeling reagent mixture of 350 mM anthranilamide (ACROS Organics), 1 M sodium cyanoborohydride (Aldrich) in acetic acid/dimethyl sulfoxide (30:70) (Merck), was added into each well. The plate was again sealed and incubated at 60 °C with agitation at 700 rpm for 2 hr.

2.4. Capillary Electrophoresis (CE) Analysis to Check Glycan Release

After a desalting step, the IgG samples, both with and without PNGase F treatment, were prepared using the ProteomLab™ SDS-MW kit (Beckman Coulter). Briefly, they were mixed with sample buffer from the kit to give a total volume of 95 µl with concentration between 0.2 and 2 mg ml^{-1}. Internal standards with 10 kDa MW and 2-mercaptoethanol were then added, followed by heating at 100 °C for 3 min. The samples were run on Beckman Coulter PA 800 Capillary Electrophoresis system with bare-fused silica (50 µm ID, total length 30.2 cm) capillary.

Prior to sample loading, the capillary was preconditioned with 0.1 M NaOH, 0.1 M HCl, water, and SDS–Gel rinse. The Capillary Gel Electrophoresis separation was achieved at −15 kV for 30 min. A summary of the steps and parameters used for the CE analysis is given in Table T2, Supporting Information.

2.5. Orbitrap Analysis of Intact and Deglycosylated IgG

For intact protein analysis, the sample was diluted to a concentration of 0.05 µg µl^{-1} and injected onto the mass spectrometry for analysis on an Orbitrap Fusion Tribrid (Thermo Scientific) coupled to a nano LC system (Dionex Ultimate 3000 RSLCnano, Thermo Scientific) with a C4 trap column, (Accucore 150-C4, 2.6 µm, 5×300 µm, Thermo Scientific). Intact protein was enriched onto the trap column using a loading solvent of 0.1% formic acid in water at a flow rate of 30 µl min^{-1} for 5 min before being eluted and separated on a 75 µm × 15 cm Accucore C4 easy spray nano column (Thermo Scientific) at a flow rate of 500 nl min^{-1} using a gradient of 15% solvent B (0.1% formic acid in 100% ACN) for 8 min, 15–95% B in 8 min, 95% B for 5 min, 95–15% B in 3 min, 15% B for 9 min. Solvent A is 0.1% formic acid in water.

Full FT-MS scan was acquired in the range of m/z 2000–4000 in standard pressure mode, with source voltage set at 2000 V, ion transfer tube temperature at 300 °C, resolution at 15 K, AGC target of 5×10^5, maximum injection time of 100 ms, RF lens at 60%, source fragmentation at 60 eV, and microscans 10.

Raw data obtained from intact protein analysis were deconvoluted using Protein Deconvolution 4.0 software (Thermo Scientific) to obtain the intact masses of the antibody with mass accuracy set at 20 ppm.

2.6. Solid Phase Extraction (SPE) Clean-Up

The labeling reaction was quenched by 200 µl of acetonitrile (Merck)/water (95:5) per well. Hypersep DIOL Plate (Thermo Scientific) was washed with 1 ml of acetonitrile (Merck)/water (95:5), 1 ml of water, 1 ml of acetonitrile /water (95:5). The labeled mixture was applied onto the Hypersep Plate. The plate was washed five times with 700 µl of acetonitrile/water (95:5) before elution with 2×200 µl water. The sample was dried and reconstituted in 50 µl of acetonitrile/water (70:30) for UPLC-MS analysis.

2.7. Analysis of Released Glycans From IgG

A total of 10 µl of the reconstituted 2-AB glycan sample was injected to the WATERS Biopharmaceutical platform with UNIFI software

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as described previously.[28] The platform consists of a coupled system of an H class Acquity UPLC and a quadrupole-time of flight (QTOF) mass spectrometer (Xevo G2-S), which is controlled by UNIFI software. The glycan mixture was separated on the HILIC column using a binary solvent system. Solvent A is 50 mM ammonium formate (pH4.4) and solvent B is acetonitrile. The analytical run takes place in 16 min by ramping up the solvent A from 30 to 47%. An external standard of 2-AB-labeled dextran ladder (Waters Corporation) was used to normalize the raw retention time of each chromatographic peak into a GU via fitting into a calibration curve.[7] The GU value of each chromatographic peak was then used to give primary assignment to glycan structures. In case of structural ambiguity, that is, a GU value corresponding to more than one structure within the error tolerance (±0.25 GU), a decision was then made based on both accurate mass confirmation (5 ppm error) by the online ESI-QTOF and the possible biosynthetic pathway of N-glycans in Chinese Hamster Ovary (CHO) cells.

3. Results and Discussion

3.1. Overview of the Workflow

The workflow shown in Figure S1, Supporting Information combines intact antibody analysis along with released glycan profiling. While the intact antibody analysis provides a fast and robust route for quickly assessing the major glycoforms of the antibody, the released glycan analysis yields additional detailed quantitative information on glycan structures including those of minor abundance which can be equally crucial for batch to batch comparison in the manufacturing process. The intact antibody analysis gives information as to how the glycan structures are paired on the two heavy chains of antibody. It can also shed light on possible modifications to the protein such as C-terminal lysine residue clipping and N-terminal glutamic acid/glutamine conversion to pyroglutamic acid. The sample preparation is performed in 96-well plate format which makes it amenable for automation on commercial liquid handling system such as Assaymap Bravo.[29] A more detailed account on the optimization for the sample preparation steps and glycan structure analysis was given in sections 3.2 and 3.3, respectively.

3.2. Optimization of Sample Preparation Steps

Optimization was performed at various steps including Protein A purification, PNGase F digestion for glycan release, 2-AB labeling, and sample cleaning by SPE plate as described in the following subsections.

3.2.1. Using MS Compatible Elution Buffer for IgG Purification

A total of 0.1 M glycine with HCl at about pH 3 has been a common choice of elution buffer used for protein A purification of IgG. However this makes the eluted sample incompatible with the following LCMS analysis unless an extra desalting step is applied. We tested the recovery rate of IgG purification using protein A plate with 0.1% Formic Acid (pH = 2.5) which is MS compatible. An average of above 90% recovery rate was achieved when an anti-HER2 antibody was used in the test while glycine elution buffer can only yield a recovery rate around 70% (**Figure 1**A).

3.2.2. PNGase F Digestion Optimization to Achieve Complete Glycan Release

A total of 200 μg of IgG (anti-HER2) was added to each well and 150–1000 U recombinant PNGase F were added for a 2 hr digestion test at 37 °C to determine the amount of enzyme needed for complete release of the glycans from the antibody. After digestion, the samples were desalted and subjected to CE analysis (section 2.4) to measure the level of deglycosylation after 2 hr incubation. When deglycosylation is incomplete, two peaks are observed corresponding to the deglycosylated and glycosylated heavy chains (Figure 1B). Using 1000 U of PNGase F, 200 μg of IgG was completely deglycosylated in 2 hr.

3.2.3. Incubation Time for 2AB Labeling

A 2-AB labeling of the PNGase F released free glycan pool was carried out at 60 °C for 30 min, 1, 2, and 4 hr, respectively (Figure 1C). The fluorescence signal response from the major glycan structure F(6)A2 was used as an indicator of the overall labeling efficiency. Two hours was chosen as the optimum labeling time.

3.2.4. Using Water to Elute Labeled Glycans in the Clean-Up Step

After 2-AB labeling, unreacted dye was removed from the sample by SPE (section 2.6). The labeled glycans were eluted with a polar solvent. In Figure 1D, it was observed that compared to 20% of ACN, water achieved more thorough glycan elution to give much higher intensity of the glycan peaks.

As summarized in Figure S1, Supporting Information the overall workflow consist of protein A purification, PNGase F digestion for 2 hr at 37 °C, 2-AB labeling for 2 hr at 60 °C and clean-up on a diol plate. The whole sample preparation takes around 6 hr and all steps are in plate formats which can be automated on an automation station equipped with vacuum manifold and shaking/heating unit.

3.3. Glycan Analysis

The glycan analysis was performed for both released glycan and intact IgG on LCMS. The released glycan analysis provides information on the detailed abundance distribution of each oligosaccharide after being released from IgG. While the intact IgG analysis detect molecules with two sugar chains attached which is also the its functioning state in the body.

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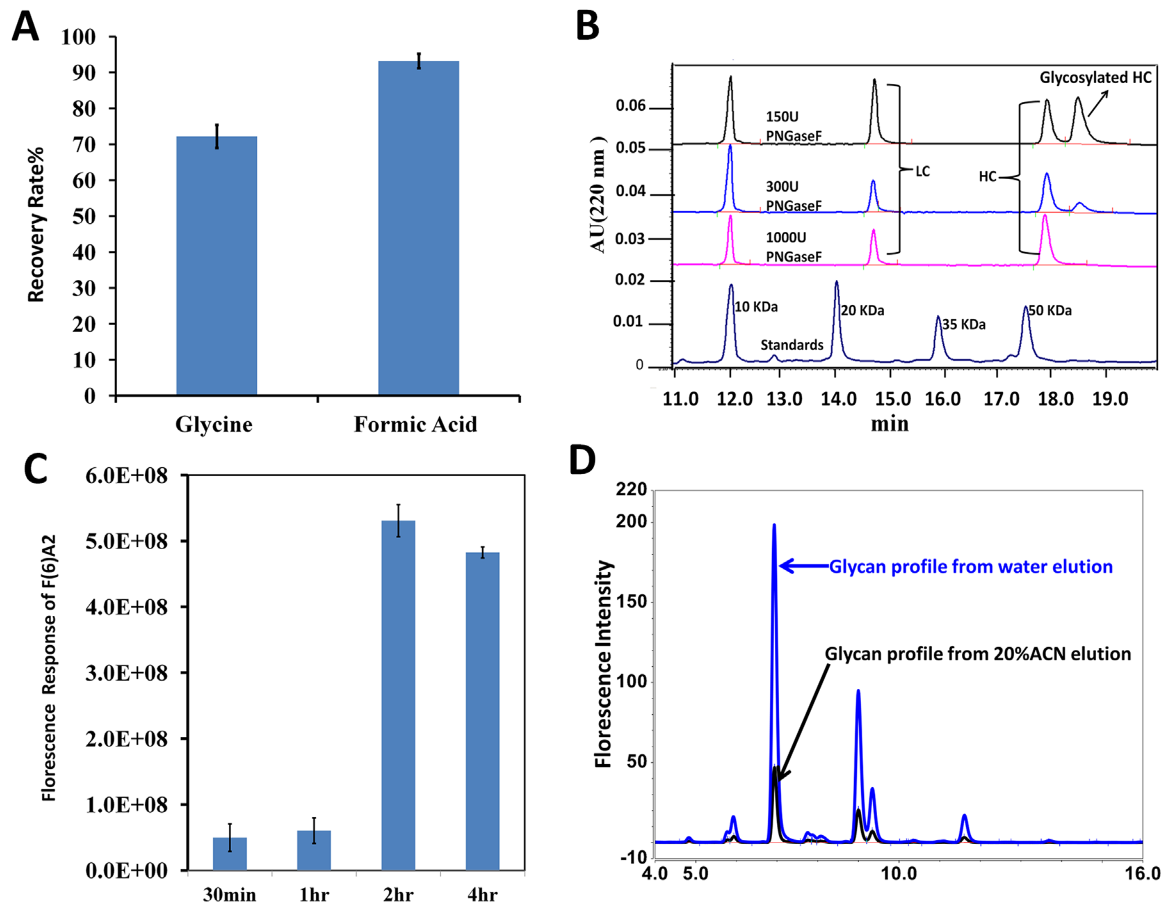

Figure 1. Optimization for various steps in the workflow: (A) Formic acid serves as a better elution buffer for IgG purification. B) Capillary Electrphoresis to check for completeness of deglycosylation by PNGase F as increasing amount of the enzyme is used to achieve thorough release of the glycan from IgG. C) Incubation time test for 2AB labeling. D) Elution buffer test for SPE clean-up step of 2-AB labeled glycans.

3.3.1. Released Glycan Analysis

The 2-AB labeled glycans were analyzed on a Waters UNIFI system. The glycan structures were assigned based on the GU values computed from the retention time on HILIC chromatography using a dextran calibration curve.[7,8] On the UNIFI system, the preliminary peak assignment was orthogonally checked by MS data. The area under each peak was normalized against total area to give the relative abundance of a particular glycan structure. Glycan profiles of the same IgG sample obtained through manual preparation (non-plate based), a commercial kit (GlykoPrep®) and our in-house 96 well based method were compared ($n = 4$ for each method) in **Figure 2**A. T1 in Supporting Information provides detailed annotation for the glycan structure names mentioned in the Figure 2A.

Coefficient of Variation (CV) was computed for each glycan species in these three methods. The in-house plate based method had an average CV of 0.12 which is comparable to the value of the manual method (CV = 0.12) while the commercial kit gave the lowest average CV at around 0.08. The CV values for the four major glycan species namely, F(6)A2, F(6)A2[6]G(4)1, F(6)A2[3]G(4)1 and F(6)A2G(4)2 all did not exceed 0.05. The minor glycan species tend to give much larger CV values potentially due to the fact that a slight difference in drawing the baseline for integration may cause significant percentage change if the peak is rather small. For fucosylation percentage (sum of relative abundance of fucosylated glycan species), the three methods gave very close values (90.96% for non-plate, 90.93% for in-house plate, and 89.61% for commercial kit).

Aliquots of an IgG sample were added into 48 wells for the released glycan analysis. The profile of relative abundance from individual wells was shown in Figure 2B for the 48 replicates with the major glycan species all having CV < 0.1. The fucosylated percentage is computed to be at 90.49% (CV = 0.01) which agrees very closely to fucosylated percentage computed for experiments in Figure 2A. This further demonstrated the reproducibility and robustness of the workflow even when we pushed for higher throughput (n-48) for testing.

3.3.2. Intact IgG Analysis Result

The intact IgG analysis on anti-HER2 IgG (both glycosylated and deglycosylated forms) is shown in (Figure S2A and S2B, Supporting Information). The deglycosylated IgG analysis (Figure S2B, Supporting Information) gave fewer peaks

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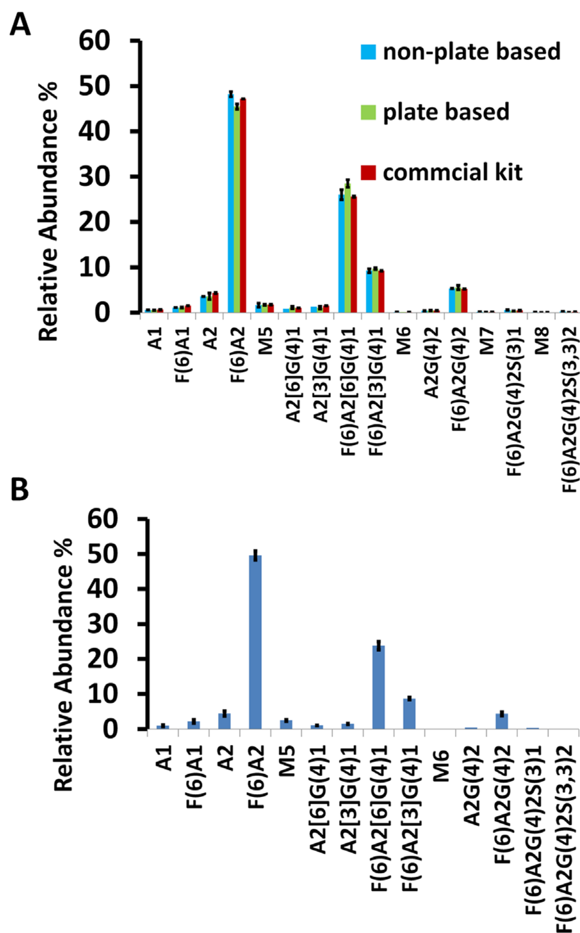

Figure 2. Released glycan analysis: (A) Glycan profile comparison for three different sample preparation methods – manual preparation without plate (blue), 96 well plate based method (green) and commercial kit (red), $n = 4$ (B) Glycan profile obtained from 48 wells of identical IgG sample. The mean value of relative abundance were computed together with standard deviation for $n = 48$.

compared to glycosylated sample (Figure S2A, Supporting Information) thus provided an easier check for modification (other than glycosylation) on the protein.

Using the deglycosylated antibody as a mass reference, the paired glycans of IgGs were inferred for anti-HER2 IgG (Figure S2A, Supporting Information). The mass error of the six intact IgG glycoforms varied between 0–4 Da with an average of 1.97 Da (13.2 ppm).

The peak heights also provided semi-quantitative measure of the abundance of the glycoforms. These glycan pairing information gave a more concise idea of the glycosylation status of the IgG molecule since it actually functions with a paired glycan structures.[30,31] Consequently, it makes more sense to consider glycan pairing when studying the structure-function relationship of IgG.[27]

In Figure S2B, Supporting Information the mass peak (145 168.6 Da) from the deglycosylated sample closely matched the theoretical mass of deglycosylated anti-HER2 IgG with no C terminal lysine residues. It also revealed a minor peak of 145 332.1 Da which is consistent with glycation, a modification which has been shown to impact on antibody clearance in a mouse model.[32]

In comparison, the dominant mass peak from deglycosylated anti-TNFα antibody (Figure S2C, Supporting Information) matched closely to the theoretical mass of the protein with both C-terminal lysines cleaved[33,34] and both glutamic acids converted to pyroglutamic acid residues at the N-terminus.[35] Carboxypeptidase B (CB) treatment of the IgG samples followed by Weak Cation Chromatography (WCX) was performed to verify the proposed C-terminal lysine clipping modifications. Figure S2D, Supporting Information showed that peaks corresponding to more basic variants in anti-TNFα sample disappeared after CB treatment.

3.4. Applications in Bioprocessing

3.4.1. Glycan Profiling for IgG Harvested at Various Time Points From Fed-Batch Culture

IgG samples (anti-HER2) were taken at six time points from the bioreactor to trace the modifications of the protein over 15 days. Around 10–15 ml of the supernatant from the fed-batch culture was harvested at day 3, 6, 8 10, 13, and 15 with viable cell density (VCD), cell viability, and IgG titers measured. Integrated viable cell density (IVCD) and specific productivity for the cells were calculated (Table S3, Supporting Information) to reflect the state of the producing CHO cells in the bioreactor at the time of sampling.

The six samples were analyzed in triplicates for both intact IgG and released glycans. The glycan species of major abundance were in the order of F(6)A2 > F(6)A2[6]G(4)1 > F(6)A2[3]G(4)1 > F(6)A2G(4)2 (total sum of abundance of the 4 species >90%), the order or relative abundance was confirmed with one-tail paired *t*-test for the three hypothesis F(6)A2 > F(6) A2[6]G(4)1, F(6)A2[3]G(4)1 > F(6)A2G(4)2, and F(6)A2[3]G(4) 1 > F(6)A2G(4)2 which yielded p values all much smaller than 0.05. The relative abundance of F(6)A2 structure reached a minimum in day 8 (**Figure 3**A).

In Figure 3B, the G0F – G0F glycoform abundance also reached a minimum in day 8. This minimum corresponded to the time when specific productivity of the cell hit the highest (S3). This observation prompted us to hypothesize that when the cells were producing IgGs at the maximum speed, its galactosylation machinery also attained highest efficiency hence more mature glycans (G1F and G2F) and less G0F was observed.

3.4.2. Glycan Profile for Clonal Selection

A panel of 15 clones was used to produce anti-TNFα IgG in 50 ml shaking flask batch cultures. The antibodies were analyzed in 96-well plate format to quickly assess its glycan profile. The relative abundance of major glycan species (F(6)A2, F(6)A2[3]G(4) 1 + F(6)A2[6]G(4)1, F(6)A2G(4)2) was computed and plotted in Figure S4A, Supporting Information. In terms of the ratio of F(6) A2: F(6)A2[3]G(4)1 + F(6)A2[6]G(4)1: F(6)A2G(4)2, clone 4 (BTI_4) has the lowest abundance of F(6)A2 and highest abundance of F(6)A2[3]G(4)1 + F(6)A2[6]G(4)1 (Figure S4A,

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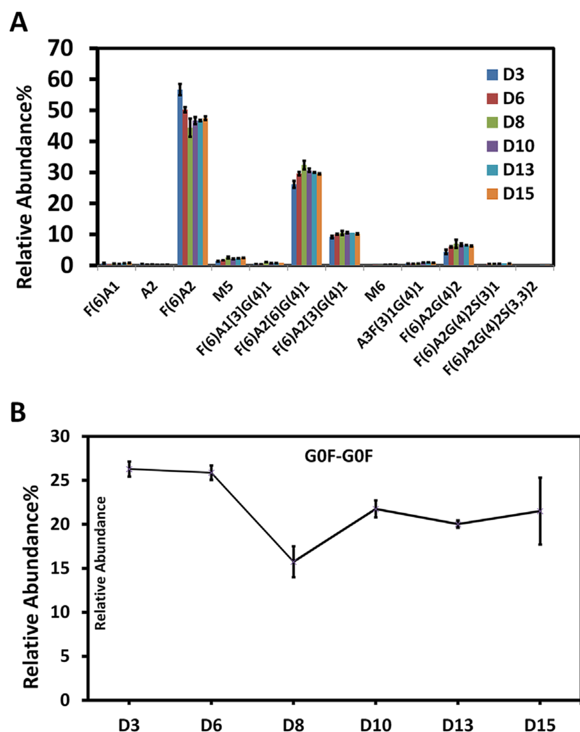

Figure 3. Glycan profile change in the Fed-Batch culture: (A) Relative abundance of glycan species from IgG sample in the bioreactor. B) G0F-G0F level variation ($n = 3$) from Intact IgG analysis.

Supporting Information). Such a trend was confirmed in the intact analysis as the G0F–G0F glycoform percentage for clone 4 was the lowest among all 15 clones (Figure S4B, Supporting Information).

In Figure S4C, Supporting Information mass peaks from deglycosylated IgG of BTI clone 5 (BTI_5) showed a major peak (145,192.6 Da) corresponding to the mass of Humira molecule with both lysine being clipped at C-terminal and two pyroglutamic acid formation at the N-terminal. The adjacent peak (145,354.5 Da) gave a 162 Da shift which can be accounted for by a glycation modification.

All these information served as a comparison basis with the reference antibody. It helps in selecting the right clones to proceed with the biosimilar development pipeline.

3.5. Conclusions

In this plate based workflow, both released glycans and intact IgG were analyzed to allow fast and detailed characterization of biopharmaceuticals on two levels. The glycan profile obtained by this workflow has been verified through comparison with a non-plate based method and a commercial kit. Forty-eight IgG samples were processed using the workflow to demonstrate its reproducibility. Trace amounts of sialylated glycan structures with relative abundance of less than 0.5% can still be detected and quantified in the workflow. While the workflow is amenable to automation on a liquid handling platform, several modifications may be attempted to improve its performance. For the protein A plate purification step, the supernatant may give foaming problem during vacuum manifold operation. To address this, a microcartridge based purification step may be considered which is easy to automate on the AssayMap Bravo system to avoid possible cross contamination among samples. Novel glycan labels such as RapiFluor-MS can be used to replace 2-AB labeling to further enhance the detection sensitivity and shorten the incubation time considerably. Availability of a GU library for glycan structures with the appropriate tag is very crucial. For samples with more challenging glycan structures, exoglycosidase enzymes should be used to further elucidate the glycan structure. Finally, though the UNIFI platform does provide an information management system, more flexible informatics tools are still needed to speed up data processing, extraction and report generation for large number of samples in exploratory research environment.

Abbreviation

2-AB, 2-aminobenzamide; AGC, Automatic Gain Control; FT, Fourier Transformed; HER, Human Epidermal growth factor Recotor; HILIC, Hydrophilic Interaction Chromatography; IgG, Immunoglobulin G; MALDI-TOF, Matrix Assisted Laser Desorption Ionization-Time of Flight; PBS, Phosphate Buffer Saline; RF, Radio Frequency; SDS, Sodium Dodecyl Sulfate; SPE, Solid Phase Extraction; TNF, Tumor Necrosis Factor; Tris, Trisaminomethane.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no commercial or financial conflict of interest. Prof. Pauline M Rudd is a Visiting Investigator supported by ASTAR's Joint Council Office Visiting Investigator Programme Grant.

Keywords

cell culture, clonal selection, intact IgG, liquid chromatography mass spectrometry (LCMS), paired glycoforms

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