U.S. FOOD & DRUG ADMINISTRATION

Biosimilar User Fee Act (BsUFA) III Regulatory Science Pilot Program

ANNUAL REPORT

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Contents

Report Overview[1]

Table 1: High-level overview of the project objective, aim(s) progress, outcomes, and timelines for communication and regulatory impact (1-2 sentence max per table cell).

Project Title:	**Establishment of A Feasible Method to Quantify Major Glycoforms of Human IgG1 mAb Drugs and their Biosimilars in Culture Media as a Component of Process Analytic Technology**
Investigator:	Tongzhong Ju
Organization:	FDA/CDER/OPQ/OPQR/ DPQRIII
Grant No. (if applicable)	N/A
Project Objective:	Develop mouse monoclonal antibodies against human non-glycosylated and glycosylated IgG1, and establish a Biolayer Interferometry (BLI) method to quantify the major glycoforms of human IgG1 mAb drugs

Specific Aim(s)	Progress	Outcomes	Communication Timeline
1. Aim 1: Further characterization of the purified mouse mAbs (mAb-a, now mAb-NG and mAb-c, now mAb-G) using differently glycosylated human IgG1 mAb drugs to determine their affinity to their specific glycoforms of human IgG1 mAb using Biolayer Interferometry (BLI) or SPR	100% 1. Completed the comprehensive characterization of the specificities of mouse monoclonal antibodies (mAbs) generated against glycosylated and non-glycosylated human IgG1. 2. Completed the Kinetics (K_D) of the mAb-NG and -G with towards their corresponding peptide and glycopeptide epitopes.	1. Mouse mAb-NG and -G antibodies specifically recognized and bound non-glycosylated and glycosylated human IgG1, respectively. 2. With the BLI method, The K_D of mAb-NG toward human IgG1 Fc-Peptide was determined at 1 nM, while the K_D of mAb-G toward the Fc glycopeptides was 1~2 nM.	1. Presented the project at the FY25 OPQ CoE ORISE Seminar Series. 2. Presented the project progress at the OPQR/DPQRIII monthly meeting. 3. Presented an abstract and poster at the FDA Science Forum-2025 on June 12th, 2025. 4. Presented a poster at the FDA/OPQ/OPQR Poster Day on June 17th, 2025.

[1] This section will be used by program for broader research portfolio and regulatory impact analysis by the BsUFA III steering committee.

Specific Aim(s)	Progress	Outcomes	Communication Timeline
2. Aim 2: Establishment of a BLI method to quantify the major glycoforms of human IgG1 mAb drugs and their biosimilars in the production media	Ongoing. 80% 1. Developed an ELISA method to measure the major glycoforms of two human IgG1 drugs. 2. Established the BLI method to quantify the major glycoforms of human IgG1 mAb drugs using mAb-G.	Both ELISA and BLI formats were applicable to quantify the major glycoforms of adalimumab, trastuzumab and a control human IgG1 mAb, consistent with the results from the mass spectrometric (MS) analysis.	Amended: Publication in a scientific journal. October 1st, 2025 - March 31st, 2026
3. Aim 3: Validation of the method using the IgG1 mAb drugs and available biosimilars	Ongoing: 50% 1. Completed the measurement of the major glycoforms of 5 trastuzumab biosimilars with the BLI method. 2. Completed the specific measurement of major glycoforms of Fc region of cetuximab which has additional N-glycosites on its Fab region.	1. The BLI method was applicable to measure the major glycoforms of IgG1 mAb biosimilars. 2. The BLI method specifically measure the glycoforms of the Fc region in the mAbs that carry more N-glycans at other region.	Amended: Showcase of the method to the Quality Assessors

Progress Summary

Monoclonal antibody (mAb) drugs, including their biosimilars, constitute nearly half of all protein drugs. IgG1 mAb drugs produced from CHO cells often contain three major glycoforms due to heterogeneous N-glycosylation at the Asn297 residue in their Fc domain: fucosylated, afucosylated, and high-mannose. N-glycosylation is a critical quality attribute (CQA) for many IgG1 mAb drugs as it directly impacts their effector functions/efficacies, such as antibody-dependent cell-mediated cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC), and pharmacokinetics/pharmacodynamics (PK/PD), and thus needs to be controlled. A small fraction of IgG1 mAb is not N-glycosylated (non-glycosylated), which lacks effector activity. Mass spectrometry (MS) and fluorescence-tagging coupled with (U)HPLC are the current tools for quantifying these glycoforms in purified IgG1 mAbs. However, these methods are not practical for biosimilar cell line development and advanced manufacturing or continuous manufacturing. To address this unmet need, we are developing a feasible, rapid, and high-throughput method to quantify the relative amount (%) of three major glycoforms of IgG1 mAb drugs using a biolayer interferometry (BLI) format. We generated and characterized mouse monoclonal antibodies, mAb-NG and mAb-G, that specifically recognize non-glycosylated (NG) and differently glycosylated (G) human IgG1 mAbs, respectively. The mAb-G requires both sugar and Fc peptide for binding with high affinity in the 1-2 nM range (K_D). We first tested an ELISA-based assay using these mouse mAbs, resulting in consistent measurement of % glycoforms of therapeutic IgG1

drugs compared to MALDI-TOF/MS analysis. When applied to a BLI platform, the % glycoforms of IgG1 mAb drugs and biosimilars were consistently measured by mAb-G. With mAb-NG, which has a K_D of 0.9 nM to the Fc peptide, we established an ELISA to quantify non-glycosylated human IgG1. Optimization and validation of the BLI method with different IgG1 mAb products and their biosimilars, as well as with harvest media, is ongoing.

Project Objective:

Develop mouse monoclonal antibodies against human non-glycosylated and glycosylated IgG1, and establish a Biolayer Interferometry (BLI) method to quantify the major glycoforms of human IgG1 mAb drugs and biosimilars.

Aim 1

Comprehensively characterize the purified mouse mAbs (mAb-NG and mAb-G) using differently glycosylated human IgG1 mAb drugs to determine their affinity to their specific glycoforms of human IgG1 mAb using Biolayer Interferometry (BLI). 1). We have successfully characterized the specificities of mouse monoclonal antibodies (mAbs) generated against glycosylated and non-glycosylated human IgG1. 2). We have successfully tested an ELISA format for measuring the affinity of the mAb-NG and -G with a human IgG1 drug (Humira) and a recombinant non-glycosylated human IgG1. Using BLI, the K_D of mAb-NG to its corresponding peptide was ~1 nM, and the K_D of mAb-G to its corresponding glycopeptide w/o core fucose was 1, and 2 nM, respectively.

Aim 2

Establish a BLI method to quantify the major glycoforms of human IgG1 mAb drugs and their biosimilars in the production media.

Aim 3

Validate the technology/method using the IgG1 mAb drugs and available biosimilars. The proposed experiments have not been conducted yet. As mentioned in Aim 2, we will test any unprocessed bulk of human IgG1 drugs and their biosimilars to quantify % glycoforms once the BLI method is established.

Research Outcomes

Aim 1: Comprehensively characterized the mouse monoclonal antibodies: 1) We have successfully characterized the specificities of mouse monoclonal antibodies (mAbs), termed mAb-G, and mAb-NG that specifically target glycosylated and non-glycosylated human IgG1, respectively. 2) We have successfully tested an ELISA format for measuring the affinity of the mAb-NG and -G with a human IgG1 drug (adalimumab) and a recombinant non-glycosylated human IgG1. 3) Using BLI, the K_D of mAb-NG and mAb-G toward their corresponding peptide and glycopeptides w/o core fucose was determined.

In the previous relevant project, we have successfully generated two different murine monoclonal antibodies by traditional immunization method: mAb-a (now termed mAb-NG) recognizes non-

glycosylated human IgG1 peptide, and mAb-c (now termed mAb-G) recognizes both aFucosylated and Fucosylated IgG1 peptides with high binding affinity on ELISA. We have obtained 50mg of purified mAb for each. Next, we investigated the respective binding of mAb-G and mAb-NG to human IgG1 drugs (adalimumab, and trastuzumab) and human IgG1 mAb controls which include the non-glycosylated human IgG1 (NG-IgG1), and the aFucosylated human IgG1 mAb, anti-βGal. By mass spectrometry (MS), we confirmed N-glycans from the human IgG1 drugs. In Western/Lectin blotting and ELISA analyses, mAb-NG specifically bound to NG-IgG1 with no cross reactivity to glycosylated IgG1s; and in contrast, mAb-G only bound to glycosylated human IgG1s, but not NG-IgG1. In an ELISA format, we calculated the affinity constant (K_D) of mAb-G to adalimumab to be about 20 nM; and mAb-NG with non-glycosylated IgG1 was determined at about 50 nM. Using Biolayer Interferometry (BLI) method, we measured the affinity constant (K_D) of mAb-NG and mAb-G with their corresponding peptide and glycopeptides at 1 nM, and 1~2 nM, respectively. Collectively, the comprehensive characterizations have demonstrated the high specificity and affinity of mAb-NG and mAb-G.

Aim 2: Both ELISA and BLI formats were established to quantify the major glycoforms of adalimumab, trastuzumab, and a control human IgG1 mAb, the quantity (%) of IgG1 glycoforms.

In an ELISA format, we quantified % glycoforms of IgG1 drugs with mAb-G, showing that the results were consistent with that from MS analysis. The procedure takes ~2 days. To establish a BLI method, we have explored several approaches and finally established the method using FAB2G biosensor which binds to the HC1 of human IgG1. The established BLI method only takes less than 2 hours to get the results. We have measured the abundance (%) of each major glycoform of human IgG1 mAb drugs and the control IgG1 mAb with high reproducibility. More importantly, the BLI results were consistent with the data obtained from the MS analysis. Additionally, we have also proved that the cell culture media did not interfere the measurement of glycoforms of IgG1 mAb drugs using the BLI method. These results and others demonstrated that the BLI method is applicable to quickly and feasibly measure the major glycoforms of human IgG1 mAb drugs. Moreover, the high-throughput nature of the BLI format makes it applicable to screen the production cell lines for human IgG1 mAb biosimilars with regard to the similarity of glycosylation, thus facilitating the development of IgG1 mAb biosimilars.

Aim 3: Validation of the method has been partly done. Further validation is ongoing.

We have demonstrated the specificity and reproducibility of the BLI method with the 5 trastuzumab biosimilars. The BLI results showed that the % of each major glycoform of 5 biosimilars and the reference product, trastuzumab was analytically comparable. Further validation, such as LLOD, LLOQ, linear range, precision is ongoing.

Regulatory Impact

The successful project has both regulatory and scientific impacts.

To industry, the technology will: 1) assist in development of production cell lines for biosimilars by quickly assessing the glycosylation patterns/glycoforms of mAb drugs in the cultivation media; 2) provide the ability to reliably quantify the levels of four major glycoforms of mAbs in unprocessed bulk media or in drug products to ensure the similarity between biosimilars and their reference

products with regard to the glycosylation; 3) function as a component of process analytical technology (PAT) to improve control strategies by optimizing upstream process through identifying critical material attributes (CMA) to produce better mAb drugs with consistent amount of aFucosylated mAb species; and 4) facilitate the development of advance manufacturing for production of mAb drugs and biosimilars with higher quality and better safety.

To Quality Assessors, the ultimate regulatory impact includes: 1) the development of a reliable method will enable the regulatory agency and reviewers from OPQ to make correct quality assessments of human IgG1 mAb drugs and biosimilars, and thus make adequate and effective risk-based decisions; 2) success of the project will facilitate the development of OPQ guidance in advanced manufacturing and quality assessment of the mAb drugs and their biosimilars with regard to glycosylation and glycoforms.

Communication and Dissemination

Table 2: Summary of communications and dissemination of information, results, outcomes, etc. related to this study.

Title	Type of Communication (e.g., poster, manuscript, presentation)	Source	Link (if available)
High-throughput measurement of major glycoforms of human IgG1 mAb drugs	Seminar	FY25 OPQ CoE ORISE Seminar Series, March 27th, 2025	N/A
High-throughput measurement of major glycoforms of human IgG1 mAb drugs	Presentation of project progress	The OPQR/DPQRIII Monthly meeting	N/A
Establishment of A Feasible Method to Quantify Major Glycoforms of Human IgG1 mAb Drugs in the Production Media as a Component of Process Analytical Technology	Abstract and poster	FDA Science Forum-2025	N/A
Establishment of A Feasible Method to Quantify Major Glycoforms of Human IgG1 mAb Drugs in the Production Media as a Component of Process Analytical Technology	Poster	FY25 OPQR Poster Day	N/A

After validation, the method for profiling the major glycoforms of IgG1 mAb drugs and their biosimilars for in-process testing and characterization will be published in a scientific journal. A workshop to demonstrate the method and data interpretation will be provided to Assessors and other stakeholders.

Scientific and Technical Challenges

No scientific or technical challenges were reported for this past year.

Next Steps

1. Measure the major glycoforms of human IgG1 mAb standards (from the NIST) in production media to further demonstrate its applicability.
2. Complete the validation of the BLI method using human IgG1 biosimilars.
3. Communicate with the Quality Assessors.
4. Continue searching for the endo-glycosidases with the specificity to cleave afucosylated complex N-glycans on human IgG1 by collaborating with the experts in the field. Even if the specific enzyme cannot be identified, we can use the amounts of total glycosylated (100%), fucosylated (%), and high mannose-glycan (%) to estimate the afucosylated complex N-glycoform (%) of IgG1 in an IgG1 mAb sample.

References

N/A