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ORIGINAL ARTICLE

Exploring Sialic Acid Variability in Innovator and Biosimilar Monoclonal Antibodies: A Comparative Study of Rituximab and Trastuzumab

Hargopal Satsangi[1], Ankita Shastri[2], Ishan Ajit Subudhi[3], Susheelendra Vaidya[3] and Abhinav Srivastava[1]*
[1] School of Biotechnology, FS University, Shikohabad, Firozabad, Uttar Pradesh, India
[2] Indian Biological Sciences and Research Institute (IBRI), Noida, India
[3] Sartorius India Pvt Ltd
*Corresponding author
Corresponding author email: abhinav.srivastava24@gmail.com

ABSTRACT

Terminal sialylation is one of the most important quality attributes (CQA) that essentially determines the protein stability and in vivo pharmacokinetics (PK) of therapeutic glycoproteins. Differences in terminal sialic acid content can arise from variations in cell line, culture conditions, and downstream processing, and may directly impact clinical performance. The present study considered the use of label-free Biolayer Interferometry (BLI) as a fast method for the relative sialic acid content screening in purified innovator monoclonal antibodies (mAbs) and their biosimilar candidates, offering a practical alternative to more labor-intensive analytical techniques. The sartorius Octet R8e system utilized Sialic Acid (GlyS) biosensors, which were pre-immobilized with a lectin exhibiting binding specificity towards terminal sialic acid residues. Two pairs of therapeutic mAbs, a rituximab innovator (Ristova) versus its biosimilar (Enfiera), and a Trastuzumab innovator (Herclon) versus its biosimilar (Canmab), were analyzed using a direct assay protocol at a concentration of 50 µg/mL in triplicate. The Ristova innovator exhibited an average relative binding signal of 0.888 nm, significantly higher than that of the Enfiera biosimilar at 0.608 nm, indicating a relative sialic acid deficit of approximately 46% in the biosimilar. Similarly, the Herclon innovator gave rise to 0.191 nm, a little more than the Canmab biosimilar at 0.155 nm (23% difference). The test precision was very high, as confirmed by the coefficients of variation (%CV) of less than 10% for all samples. In general, the findings presented here confirm the GlyS BLI assay as an efficient, precise, and high-throughput tool that can identify significant changes in terminal sialylation, thus being instrumental in biopharmaceutical early-stage development as well as in biosimilar comparability assessments.

***Keywords**: Sialic Acid, Rituximab, Trastuzumab, Monoclonal Antibodies*

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INTRODUCTION

Glycosylation as a Critical Quality Attribute Glycosylation is one of the most widespread and structurally complex post-translational modifications (PTMs) of therapeutic proteins [1] [2] [3]. It is a modification that changes in protein folding, conformation, solubility, isolation and purification consistency, stability, and biological activity are all influenced by glycosylation [4] [5]. Due to its intricacy and reliance on cell-line related factors and culture conditions, protein glycosylation is a Critical Quality Attribute (CQA) that is very closely monitored [6] [7]. Sialic acid is most commonly found at the non-reducing termini of N- and O-linked glycan chains and is a pharmacologically important glycosylation motif [8] [9]. Research shows that elevated sialic acid levels are in general associated with increased stability and these residues have a vital role in lessening the clearance rate of drug molecules from biological systems, thus affecting serum half-life [10] [11]. Besides PK effects, the changes in glycan structures, such as the elimination of fucose residues, may result in the increase of antibody-dependent cellular cytotoxicity (ADCC) [12] [13].

Therefore, first of all, the glycosylated CQA's (GCQAs) identification, characterization, and control, which include also the number and distribution of sialic acid residues, are the keys to product safety and efficacy [14] [15]. Analytical Challenges in Biosimilar Development Analytical challenges of biosimilar development are a topic in itself [16]. To prove that biosimilars are highly similar to the originator products, they have to be subjected to rigorous analytical testing [17]. However, despite independent cell lines and processes, biosimilars should be the same in essence as the original products [18] [19]. Glycosylation patterns are particularly susceptible to variability caused by production process inconsistencies or changes in the host cell machinery [20]. Regulatory pathways require to be equipped with analytical data in full in order to allow them to give the go-ahead for similarity of carbohydrate moieties in biosimilars and reference products [21] [22].

High-resolution standard methods for glycan analysis such as High-Performance Liquid Chromatography (HPLC), Mass Spectrometry (MS), and Liquid Chromatography-Mass Spectrometry (LCMS) are generally employed to obtain detailed characterization and final product release [22] [23] [24]. However, these methods are essentially time-consuming and involve a series of steps for sample preparation, thereby making them less viable for a high-throughput approach to early process development and cell line selection [25]. The biopharmaceutical industry is in need of simpler, faster, and complementary analytical platforms that can provide actionable CQA data early in the workflow and hence, facilitate the rapid funneling of protein candidates with high potential [26] [27] [28].

APPLICATION OF BIOLAYER INTERFEROMETRY FOR GLYCAN SCREENING

Biolayer Interferometry (BLI), using the sartorius Octet platform, offers a rapid and label-free solution for molecular screening [29]. The Sialic Acid (GlyS) Kit is specifically designed for the relative screening of terminal sialic acid content in purified and crude samples [30]. The GlyS biosensors are pre-immobilized with a lectin that targets terminal sialic acid, demonstrating a binding specificity towards both N- and O-linked terminal sialic acid, with a documented higher preference for O-linked structures [31].

This assay is intended exclusively for relative screening, ranking proteins as having high, medium, or low terminal sialic acid content, and is not designed for absolute quantitation. By measuring the difference in binding signal (Δnm) between samples, the GlyS kit provides an effective measure of relative sialylation status [32].

This study aimed to apply this label-free BLI methodology to evaluate its capacity and precision for the comparative screening of terminal sialic acid levels across two pairs of purified innovator mAbs and their corresponding biosimilars.

MATERIAL AND METHODS

CHEMICAL AND REAGENTS

Instrumentation and Software

All measurements were performed using an Octet R8e Biolayer Interferometry System Sartorius. Data acquisition and analysis were performed using Octet Data Acquisition and Data Analysis software version 13.1.0.38. The hardware utilized an 8-channel sensor capability in the experiments. Black polypropylene 96-well microplates were used for the assays.

GlyS Kit Components and Auxiliary Reagents

The Sialic Acid (GlyS) Kit (Sartorius) provides the specialized components necessary for the assay:

- **GlyS Biosensors**: Pre-immobilized with lectin for terminal sialic acid detection.
- **Glycan Buffer A**: Used for biosensor pre-hydration.
- **Glycan Sample Prep Buffer**: Essential diluent specially formulated to relax glycoprotein structures and maximize the accessibility of terminal glycans to biosensors.
- **Glycan Wash Buffer**: Used for washing steps.
- **Auxiliary Materials**: PBS buffer (azide-free) was used for general purposes, and Kinetics Buffer 10X (Sartorius) is generally required for the Octet systems.

Biotherapeutic Samples

Sample Category	Sample ID	Sample Info (Lot/ID)	Drug Class
R-mab Innovator	Ristova	H0349B01	Rituximab
R-mab Biosimilar	Enfiera	RA2323A	Rituximab
T-mab Innovator	Herclon	H5174B01	Trastuzumab
T-mab Biosimilar	Canmab	BI23003476	Trastuzumab

The study involved two pairs of purified monoclonal antibodies (mAbs). The innovator and biosimilar samples for each pair were tested side by side using the same method and conditions.

Sample Preparation

Protocol Rationale and Buffer Utilization

The study utilized the Direct Assay protocol because all biotherapeutic samples (Ristova, Enfiera, Herclon, and Canmab) were available in a purified form [32]. This approach simplifies the workflow by eliminating the signal amplification step, which is otherwise necessary for crude samples to selectively amplify the signal from the Protein of Interest (POI) over competing host cell glycoproteins [33].

To achieve the best results, any purified sample was required to be diluted with Glycan Sample Prep Buffer. Such a dilution, which was indicated to be at least 1:10 (v/v), is very important as the buffer guarantees the unfolding of the glycoprotein structure, thus making the terminal sialic acid residues more accessible to the GlyS lectin on the biosensor surface [32].

Assay Setup and Concentrations

GlyS biosensors were prepared by pre-hydrating them in Glycan Buffer A for a minimum of 10 minutes prior to the assay start. The instrument temperature was maintained at approximately $29.8 \circ C$ throughout the runs.

Two distinct concentration studies were performed:

1. **Linearity Assessment:** A dilution series (100 μg/mL, 10 μg/mL, and 1 μg/mL) was prepared for all four mAbs to confirm the appropriate dynamic range and identify a suitable concentration that avoids signal saturation.
2. **Comparative Analysis:** Based on the linearity check, a primary working concentration of 50 μg/mL was selected for the final, rigorous comparison of the innovator and biosimilar products, with each sample tested in three independent replicates.

Direct Assay Workflow

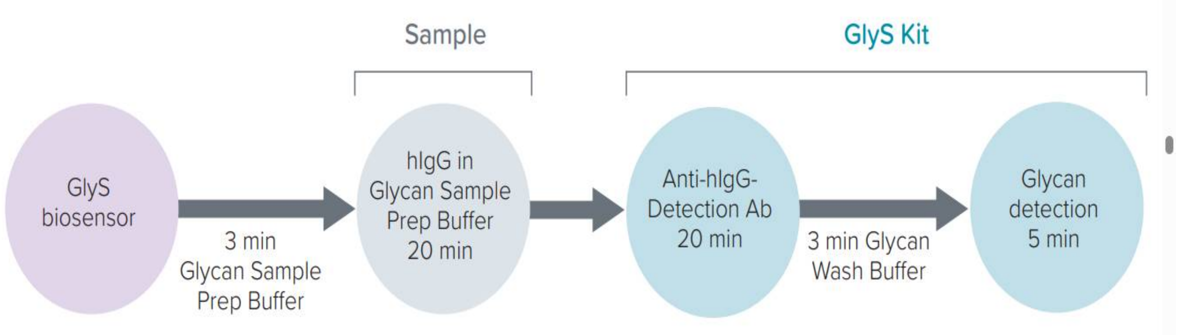

Figure 1: Assay workflow

The Direct Assay workflow involved minimal steps:

1. **Baseline:** The GlyS biosensor was exposed to Glycan Sample Prep Buffer for 3 minutes to establish a stable baseline.
2. **Sample Association:** The biosensors were then dipped into the purified POI sample (prepared in Glycan Sample Prep Buffer) for a duration of 20 minutes. This 20-minute period is recommended to ensure the binding reaction reaches saturation, providing a reliable endpoint signal (ReportPoint) for relative comparison.

Buffer samples were included in designated wells (D4 and H4 for triplicates; D11 and H11 for dilution series) to serve as zero or reference samples. The recorded signal from these reference wells was averaged and subtracted from the sample readings during data processing to eliminate non-specific binding and instrument drift.

Analysis

Instrumentation and Method Parameters

The Octet BLI system was configured to run in **Quantitation** mode, specified further as **Advanced Quantitation.** Key operational parameters were standardized across all experiments to ensure comparability:

Parameter	Setting (Triplicate Assays)	Unit/Notes
Instrument Type	Octet R8e	BLI Platform
Experiment Temperature	≈29.8	∘C
Flow Rate (Shaking)	1000	RPM (Used for all fluidic steps)
Assay Time (Total)	1379 to 1439	seconds
Read Time (Analysis Endpoint)	1139.8	seconds
Start Delay	600 or 800	seconds (For temperature equilibration)
Sensor Type	GlyS (Sialic Acid)	Lectin-immobilized biosensor

Table 1: Instrumentation and method parameters for GlyS BLI triplicate assays- Summary of Biolayer Interferometry (BLI) instrument settings and operational parameters used for triplicate relative sialic acid screening experiments performed on the Octet R8e platform. All parameters were held constant across samples to ensure analytical consistency and comparability.

The flow rate was maintained at 1000 RPM for the assay, regeneration, and neutralization steps. A significant delay was implemented at the start of the run (up to 800 s) to ensure the microplates reached the stable $30\circ C$ set temperature before the assay commenced.

Data Processing and Quantification Metrics

Data analysis was performed using the Octet Analysis Studio software. The analytical procedure adhered to established pre-processing and quantification steps:

1. **Reference Subtraction:** The primary pre-processing step involved subtracting the average response of the designated buffer wells from the raw binding signal of each sample well (e.g., $p1A4$−Average($p1D4$,$p1H4$)). This crucial step normalized the data against background noise and non-specific binding, yielding the true relative binding signal (Δnm) attributed to terminal sialic acid capture.
2. **Binding Metric Selection:** The relative sialic acid content was calculated using the **ReportPoint** metric (Binding Rate Equation). This metric measures the binding signal (Δnm) attained at a specific endpoint of the association phase, providing a direct, relative measure of captured sialic acid residues.
3. **Standard Curve Fitting:** Although this assay is for relative comparison, the quantitative data processing utilized a **Linear Point to Point** standard curve equation.

The quality of the analytical data was evaluated based on precision metrics. The Coefficient of Variation (%CV) of the triplicate measurements served as the primary indicator of assay reproducibility and robustness for comparative screening.

RESULTS AND DISCUSSION

Assessment of Linear Dynamic Range

Prior to the triplicate comparison, a dilution series was run to ensure the selected test concentration (50 μg/mL) resided within the linear dynamic range and to characterize the general sialylation levels of the four biotherapeutics [34] [35]. The results of the dilution series are presented below, showing the background-subtracted binding signal (Δnm).

Relative Binding Signal (Δnm) from Dilution Series:

Sample ID	**100 μg/mL Binding (nm)**	**10 μg/mL Binding (nm)**	**1 μg/mL Binding (nm)**
Ristova (Innovator R-mab)	1.170	0.363	0.089
Enfiera (Biosimilar R-mab)	0.700	0.272	0.101
Herclon (Innovator T-mab)	0.230	0.138	0.008
Canmab (Biosimilar T-mab)	0.243	0.140	0.045

Table 2: Relative GlyS BLI binding responses from dilution series- Background-subtracted GlyS lectin binding signals (Δnm) obtained for rituximab and trastuzumab innovator and biosimilar samples across a concentration range of 1–100 μg/mL. The data were used to assess assay linearity and to confirm that the selected working concentration (50 μg/mL) lies within the linear dynamic range for reliable relative comparison of terminal sialic acid content.

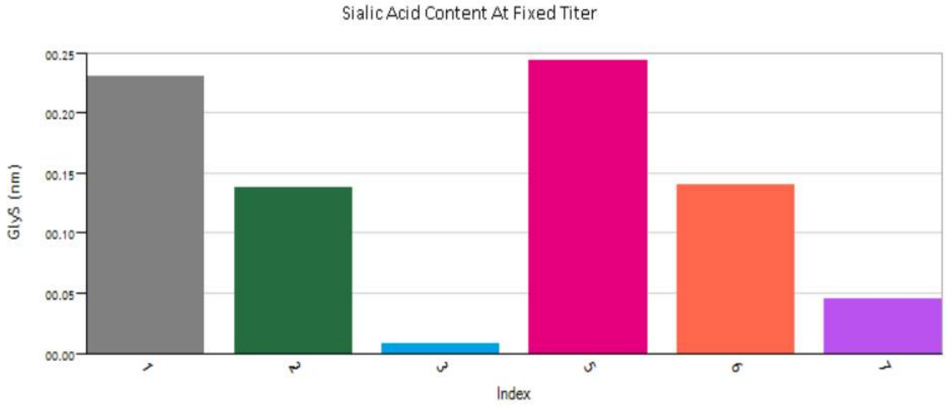

Figure 2: Sialic acid content at fixed titer for trastuzumab samples: Bar-chart representation of background-subtracted GlyS lectin binding (Δnm) at a fixed titer, illustrating relative terminal sialic acid content across trastuzumab samples [36]. The innovator product shows consistently higher binding compared to the biosimilar, indicating greater abundance and/or accessibility of terminal sialic acid residues [37].

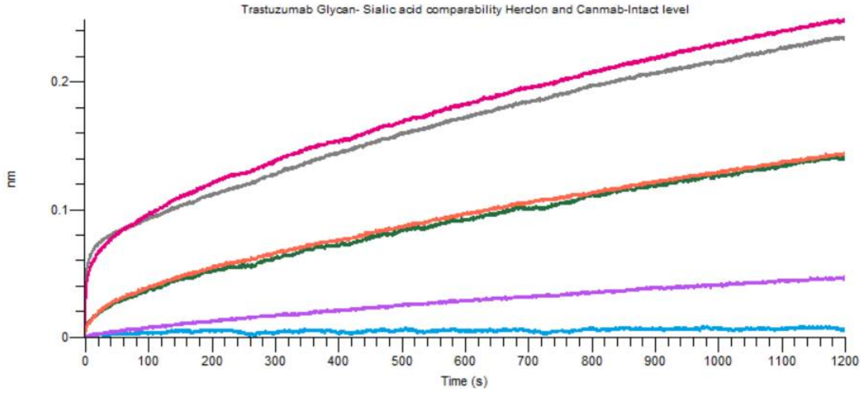

Figure 3: Intact-level BLI association sensorgrams for trastuzumab innovator and biosimilar: GlyS BLI association sensorgrams comparing trastuzumab innovator (Herclon) and biosimilar (Canm ab) at the intact level. Curves demonstrate clear separation throughout the association phase, with the innovator exhibiting higher binding despite lower absolute signal magnitude relative to trastuzumab [38] [39].

A key observation across all tested samples is that the maximum binding signal (1.170 nm for Ristova at 100 μg/mL) is orders of magnitude lower than the 60 nm signal threshold cited by the manufacturer, beyond which the algorithm may produce less accurate or "crashed" results. The low magnitude of the signals confirms that the assay was performed within the appropriate linear range, ensuring the reliability of the relative comparisons [32] [40] [41].

The data reveals a stark difference in the inherent sialylation profile between the two drug classes. The Rituximab pair (R-mab) exhibits substantially higher overall terminal sialic acid content and a broader analytical dynamic range compared to the Trastuzumab pair (T-mab) [42] [43]. As an example, the highest sialylated T-mab sample (Canmab at 100 µg/mL, 0.243 nm) just reaches a part of the signal produced by the R-mab innovator, thus indicating that there might be significant differences either in their inherent glycosylation structures or in the accessibility of the terminal sialic acid residues to the lectin [44] [45].

Relative Sialic Acid Comparison: Rituximab Innovator vs. Biosimilar

The comparative analysis of Ristova (Innovator) and Enfiera (Biosimilar) at 50 μg/mL revealed a pronounced difference in their terminal sialic acid content [46] [47].

Relative Sialic Acid Binding Comparison: Rituximab Innovator vs. Biosimilar (50 μg/mL)

Sample ID	Replicate 1 (nm)	Replicate 2 (nm)	Replicate 3 (nm)	Average Binding (nm)	Std Dev (nm)	% CV
Ristova (Innovator)	0.865	0.891	0.907	0.888	0.021	2.388
Enfiera (Biosimilar)	0.608	0.596	0.621	0.608	0.013	2.055

Table3: Relative GlyS BLI binding responses for rituximab innovator and biosimilar at 50 µg/mL - Background-subtracted GlyS lectin binding signals (Δnm) measured in triplicate for Ristova (innovator) and Enfiera (biosimilar). Average binding, standard deviation, and percent coefficient of variation (%CV) are shown to assess assay reproducibility and relative sialic acid content.

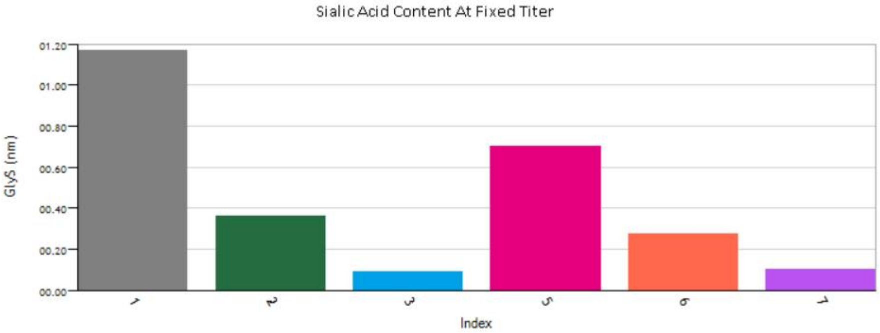

Figure 4: Intact-level BLI association sensorgrams for rituximab innovator and biosimilar: Overlay of GlyS BLI association sensorgrams for rituximab innovator (Ristova) and biosimilar (Enfiera) at the intact level [48]. The innovator exhibits substantially higher binding across the full association period, reflecting increased terminal sialic acid content [49].

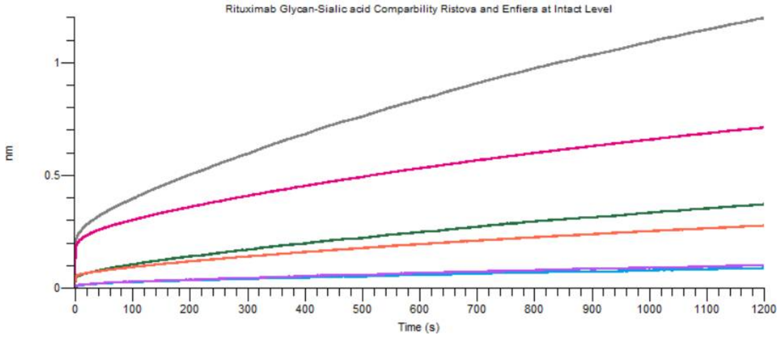

Figure 5: Sialic acid content at fixed titer for rituximab samples: Background-subtracted GlyS binding responses (Δnm) at fixed titer for rituximab innovator and biosimilar products [50] [51]. The innovator shows higher relative terminal sialylation, while overall signal magnitudes remain lower than those observed for rituximab products [52] [53].

The Ristova innovator was the source of an average binding signal of 0.888 nm, which clearly showed a strong presence of terminal sialic acid. On the other hand, the biosimilar Enfiera generated an average signal of 0.608 nm. Thus, the biosimilar has about 31% less relative sialic acid content, or the innovator can be said to have 46% more signal than the biosimilar. Such a quantitative difference in one of the most critical therapeutic CQA strongly indicates a significant difference in the glycoform profiles of the two products [54] [55].

The assay was very precise, the %CV Ristova being 2.388% and the %CV Enfiera 2.055%. The close grouping of the replicate data is a confirmation of the BLI method's accuracy in resolving these differences [56] [57]. As sialylation is the factor that directly influences serum half-life, this 46% relative shortfall of the terminal sialic acid signal in Enfiera suggests that the in vivo clearance may be faster than in the case of the innovator [58]. Therefore, a finding of this nature requires immediate demonstration of high-resolution structural details by orthogonal analytical techniques before making functional and clinical implications [59] [60].

Relative Sialic Acid Comparison: Trastuzumab Innovator vs. Biosimilar

The comparative analysis for the Trastuzumab pair (Herclon vs. Canmab) was also performed at 50 μg/mL, albeit against a lower overall baseline of inherent sialylation [61] [62] [63].

Relative Sialic Acid Binding Comparison: Trastuzumab Innovator vs. Biosimilar (50 μg/mL)

Sample ID	Replicate 1 (nm)	Replicate 2 (nm)	Replicate 3 (nm)	Average Binding (nm)	Std Dev (nm)	% CV
Herclon (Innovator)	0.179	0.201	0.192	0.191	0.011	5.801
Canmab (Biosimilar)	0.167	0.160	0.139	0.155	0.015	9.381

Table 4: Relative GlyS BLI binding responses for trastuzumab innovator and biosimilar at 50 µg/mL. - Background-subtracted GlyS lectin binding signals (Δnm) measured in triplicate for Herclon (innovator) and Canmab (biosimilar). Average binding, standard deviation, and percent coefficient of variation (%CV) are reported to evaluate assay reproducibility and compare relative sialic acid content.

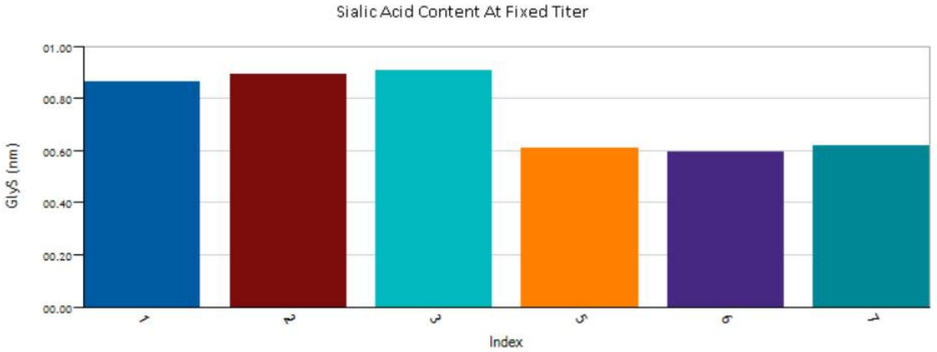

Figure 6: Summary of relative sialic acid content across innovator and biosimilar mAbs: Fixed-titer GlyS binding responses summarizing relative terminal sialic acid content across both monoclonal antibody classes. Data highlight pronounced differences for rituximab and moderate but consistent differences for trastuzumab, supporting the utility of GlyS BLI as a rapid screening tool for biosimilar comparability [64].

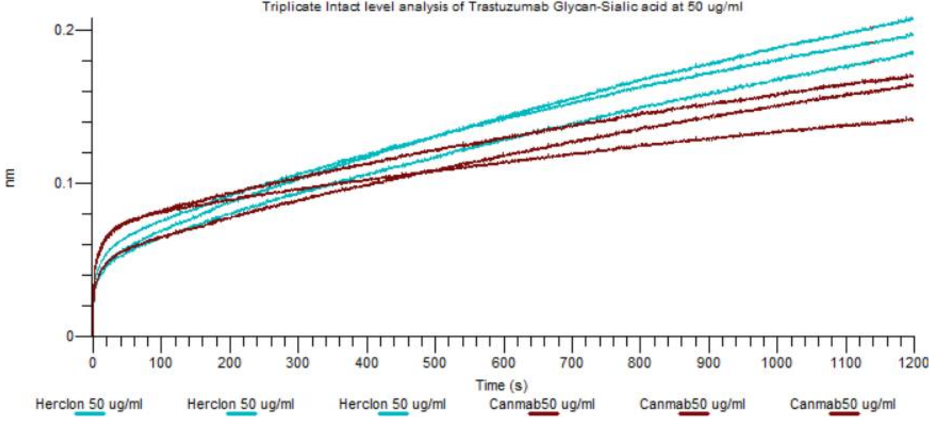

Figure 7: Triplicate intact-level comparison of trastuzumab innovator and biosimilar: Comparison of triplicate BLI association sensorgrams for trastuzumab innovator (Herclon) and biosimilar (Canmab). The innovator shows higher and more consistent binding, whereas the biosimilar exhibits slightly increased variability, consistent with calculated %CV values [65].

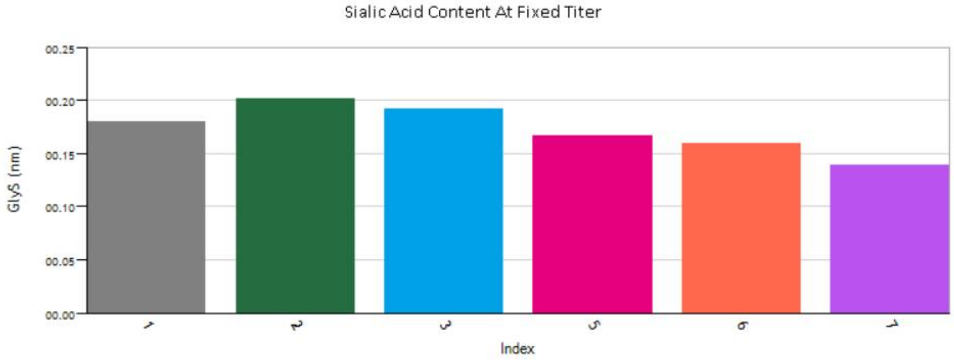

Figure 8: Summary of relative sialic acid content across innovator and biosimilar mAbs: Fixed-titer GlyS binding responses summarizing relative terminal sialic acid content across both monoclonal antibody classes. Data highlight pronounced differences for rituximab and moderate but consistent differences for trastuzumab, supporting the utility of GlyS BLI as a rapid screening tool for biosimilar comparability [64].

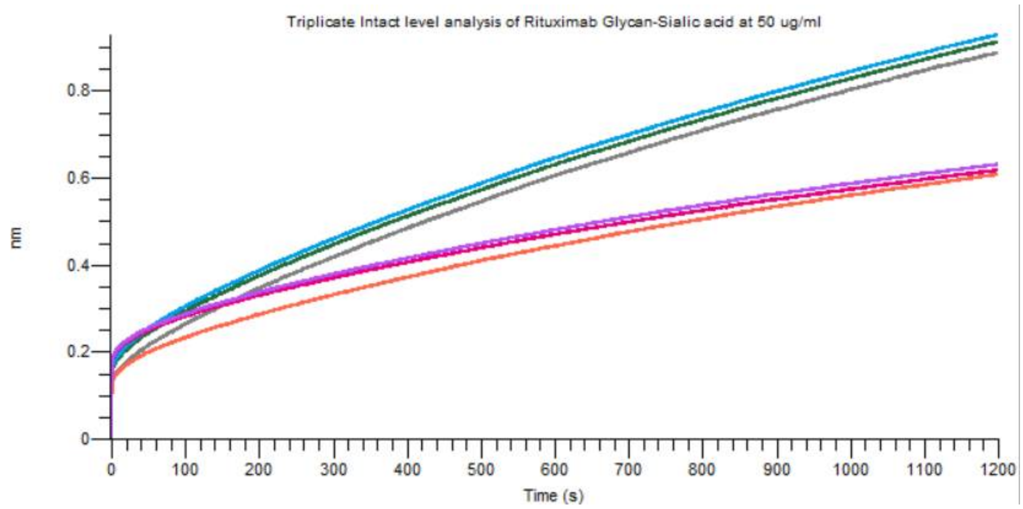

Figure 9: Triplicate intact-level comparison of rituximab innovator and biosimilar: Expanded view of triplicate BLI association sensorgrams comparing rituximab innovator (Ristova) and biosimilar (Enfiera). Clear and reproducible separation between products is observed, confirming a substantial difference in terminal sialylation [66] [67].

The innovator Herclon showed an average relative sialic acid content of 0.191 nm, which was 23% higher than the Canmab biosimilar at 0.155 nm. Although the absolute signal magnitudes were lower for the R-mab pair, the relative difference is still very obvious [68] [69].

Precision parameters were for the most part good; Herclon had a CV of 5.801%. On the other hand, the Canmab biosimilar had a higher CV of 9.381%. A detailed view of the Canmab replicates shows that one value (0.139 nm) is significantly different from the other two (0.167 nm and 0.160 nm). The overall CV is still below the 10% analytical threshold usually recognized, but this increased variability compared to the innovator indicates that there may be subtle inconsistencies either in the homogeneity of the biosimilar sample or due to the increased variability of the low-magnitude signals [70]. This point serves to illustrate that the BLI technique is very sensitive even when dealing with proteins that have intrinsically low sialylation [71].

General Discussion on Screening Utility

The observed differences in terminal sialic acid content between the innovator and biosimilar products are significant and consequential for biosimilar comparability assessments. Given that the GlyS lectin displays high affinity towards terminal sialic acid, with particular preference for O-linked structures, the measured binding signal reflects the relative abundance and accessibility of specific sialylated glycoforms [72] [73].

The utility of the GlyS BLI assay lies in its speed and capacity. Completing a direct assay for a full 96-well plate requires approximately 51 minutes [74]. This rapid throughput, combined with the demonstrated precision (low CVs), allows manufacturers to monitor CQA targets efficiently [75]. By identifying early process divergence leading to differences such as the observed 46% relative deficit in the R-mab biosimilar, the method enables a risk-based resource allocation, directing more complex and time-consuming high-resolution analytical techniques toward molecules that have already shown a critical variance [76].

Furthermore, it is recognized that for meaningful comparability data between early-stage BLI screening and late-stage HPLC/MS characterization, the latter must employ total glycan digestion methods (e.g., acid hydrolysis) that release both N- and O-linked glycans, ensuring the comparison is structurally relevant to the terminal sialic acid residues detected by the GlyS lectin [77] [78] [79] [80].

CONCLUSION

The results from this study exemplify how a label-free Biolayer Interferometry (BLI)–based GlyS lectin assay can serve as a fast and consistent method for relative screening of terminal sialic acid content in therapeutic monoclonal antibodies. It was possible to distinguish clearly the differences between the innovator and biosimilar products for both rituximab and trastuzumab by a direct assay format at 50 µg/mL. All the measurements were made within the linear dynamic range and had low coefficients of variation, which is indicative of the method being robust from an analytical point of view. This approach has the potential to be an early-stage screening tool that can detect large and moderate differences in sialylation between the products and, thus, it can be a great tool that precedes and complements high-resolution glycan characterization in biosimilar comparability studies.

FUTURE SCOPE

Future work may extend this approach to multi-batch and process-change evaluations and integrate BLI screening with orthogonal HPLC and MS-based glycan analyses. Expansion to additional lectin assays could enable broader glycosylation profiling, strengthening early risk-based decision-making in biopharmaceutical development.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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