Research Article

Comparative Glycopeptide Analysis for Protein Glycosylation by Liquid Chromatography and Tandem Mass Spectrometry: Variation in Glycosylation Patterns of Site-Directed Mutagenized Glycoprotein

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1. Introduction

Glycosylation involves the covalent attachment of glycans to specific amino acid residues on protein and is one of the important posttranslational protein modification processes [1]. Glycosylation alters the properties of proteins including pharmacokinetics, effector functions, solubility, and stability [2]. Aberrant glycosylation in glycoproteins has been related to the occurrence and progression of certain diseases [3]. Careful observation of protein glycosylation is crucial for the development of stable and effective drugs. Furthermore, they are crucial in comparing biosimilar products to reference drugs, as mandated by regulatory agencies [4]. Changes in manufacturing process conditions for biologics, such as process optimization, scale-up production, and site changes, may impact glycosylation patterns of the resulting recombinant antibody (rAb) [5, 6]. As such, glycosylation is considered a critical quality attribute (CQA) for rAb therapeutics as it has the potential to determine whether the biosimilar candidate is highly similar to the reference drug. The glycosylation pattern of a rAb affects a wide spectrum of biological processes. Therefore, consistent glycosylation is necessary to prove the drug’s ability to maintain safety and efficacy [7].

Mass spectrometry is a core technology in the field of proteomics with high-throughput performance and accurate digitalized informatics [8]. One particular mass analysis technique is matrix assisted laser desorption/ionization mass spectrometry (MALDI-MS) equipped with time-of-flight (TOF) analyzer [9–12]. Another widely used method in mass spectrometry is liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) [13–17]. LC-ESI-MS/MS is a robust, high-throughput method
in identifying and quantifying glycopeptides in enzymatic digests from various proteomic samples. High-throughput ESI-MS/MS for glycopeptides, initially separated by LC, allows for the identification and quantification of all detectable features, which, in turn, provides a more detailed account of protein glycosylation patterns. Data analysis for large amounts of raw mass data resulting from LC-ESI-MS/MS for the glycoproteome is a challenging task; therefore, various search engines have been developed such as Glycomaster DB [18], Byonic [19], MAGIC [20], and Glycoproteome Analyzer (GPA) [21]. GPA is capable of identifying site-specific N-glycopeptides efficiently and features the use of 3-top monoisotopic mass peak intensity of glycopeptides [21, 22]. The high-speed mapping of glycopeptides using GPA has proven to display analytical efficiency with a false display rate (FDR) ≤1%.

Here, we have introduced a practical method for mapping and comparing the glycosylation patterns in rAb glycoproteins, in which glycopeptide samples prepared by in-solution or in-gel protein digestion are analyzed by LC-ESI-MS-MS. The developed method is applied for comparative analysis of glycosylation patterns of biosimilar rAbs as well as a mutagenized rAb glycoprotein.

2. Experimental

2.1. Reagents and Chemicals. The reference rAb glycoprotein (Adalimumab, commercially known as Humira) was obtained from G Sam Hospital (Gyeonggi-do, Korea). HiTrap Mabselect SuRe columns were purchased from GE Healthcare and C_{18} trap column was purchased from Harvard Apparatus (Holliston, MA USA). Trypsin for protein digestion was obtained from Promega (Madison, WI, USA). 1,4-dithiothreitol (DTT), iodoacetamide (IAA), trifluoroacetic acid (TFA), and formic acid (FA) were purchased from Sigma Aldrich (St. Louis, MO, USA). HPLC-grade water and acetonitrile (ACN) were purchased from J.T. Baker (Phillipsburg, NJ, USA). CHO-k1 cells were purchased from ATCC (Manassas, VA, USA) and ExpiCHO-s cells were purchased from Thermo Fisher Scientific (Waltham, MA, USA).

2.2. Expression Vector for the Biosimilar. The light chain and heavy chain genes for biosimilar rAb were synthesized (Genscript, Piscataway, NJ, USA) and cloned into a custom expression vector, pCPp2-CMV. The expression vector contains both the heavy chain and the light chain genes for the biosimilar rAb controlled by the human CMV promoter, respectively. The vector also contains puromycin-resistance gene as a selectable marker under SV40 promoter.

2.3. Transient Expression of Biosimilar rAbs. For the transient expression of biosimilar rAbs, ExpiCHO-s cells were transfected with the expression vector and maintained in fed-batch culture following manufacturer’s protocol for max titer. Briefly, 50 ml of Expi-CHO-s cells cultured in ExpiCHO culture medium in a 250 shaker flask was transfected with 50 μg of the expression vector DNA using ExpiFectamine CHO reagent. Transfected cells were maintained on shaker at 32 °C in a CO\textsubscript{2} incubator and ExpiCHO enhancer and feed were added as recommended. Cells were monitored every 24 hours for the cell growth and viability. Cells were harvested 7-10 days after transfection before cell viability drops below 75%. Cell supernatant was collected by centrifugation at 3,000 g for 30 min and filtered through a vacuum filtration unit (0.22 um). Filtered cell supernatant was subjected to purification for biosimilar rAbs by protein A affinity chromatography using HiTrap Mabselect SuRe columns.

2.4. Mutagenesis of Biosimilar rAb Generation. A mutagenized heavy chain gene containing tryptophan to valine substitution within the CH2 domain of the heavy chain (at position C41 by IMGT codon numbering or 290 by Kabat numbering) was synthesized and cloned into the expression vector containing the light chain gene. The newly cloned DNA was transfected into CHO-k1 cells and mutagenized rAb was purified from cell supernatant using HiTrap Mabselect SuRe columns.

2.5. Stable Expression of the Biosimilar rAbs after Cell Line Generation. Cell lines for the biosimilar rAbs were generated using suspension-adapted CHO-k1 cells. CHO-k1 cells were preadapted in serum-free suspension culture and maintained in CDM4CHO (Hyclone, Little Chalfont, UK) chemically defined medium. 20 μg of the wild-type expression vector was used to transfect 2 × 10^8 cells by electroporation following a proprietary recombination-based transfection protocol. Transfected cells were monitored daily and subject to media change and antibiotic selection with puromycin (8 μg/mL) every 48 hours for 12-14 days. Stably transfected cell pool was then used for the selection of high titer cell lines by FACS cell sorting using MoFlo-XDP (Beckman Coulter, Brea, CA, USA). FACS cell sorting was performed after staining the cells using R-PE conjugated F(ab’)\textsubscript{2}, fragment goat anti-human IgG Fcγ fragment specific antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) following the protocol in Brezinsky et al. (2003) [23]. High titer cell lines were sorted directly into 96-well plates containing 200 μL of proprietary single cell growth medium per well, based on the R-PE fluorescence expression levels, and after excluding dead cells and doublers. Cell sorting was performed using the single cell sort mode to increase the efficiency sorting one cell per well. Cells sorted into 96 well plates were monitored for cell growth and clonality by scanning the plate using Clone Select Imager (Molecular Devices, San Jose, CA, USA). Approximately 15 days after the sorting, expression levels of the biosimilar rAb were determined with an ELISA assay and 20 clones with the highest expression levels were expanded for further analysis. A final clone with the highest cell titer was selected from the 20 clones, preserved in liquid nitrogen stocks, and then used for the expression of the biosimilar in a fed-batch condition. For the expression of the biosimilar from the cell line, cells were seeded in 30 ml of CDM4CHO with 8 μg/ml puromycin and 800 μL of Sheff Pulse II (Kerry Bioscience, Ithaca, NY, USA) feeding medium was added to the culture every 48 hours. Cells were
maintained at 32°C for 10-12 days and harvested when cell viability dropped below 75%. Biosimilar rAb was purified from the cell culture supernatant using HiTrap Mabslect SuRe protein A columns.

2.6. Protein Quantitation with Indirect ELISA Assay. 96-well plates for ELISA assay were prepared by coating the wells with goat anti-human IgG whole molecule primary antibody (5 μg/mL, Sigma Aldrich) and blocking with tris-buffered saline with BSA pH 8. Purified IgG from human serum was used as a standard in serial dilutions in two folds starting from 50 ng/ mL. Biosimilar rAb samples were diluted so that the titer estimation can be made within the standard curve range. Standards and samples were added to the wells in duplicates. After extensive washing, the wells were probed with goat anti-human IgG- (gamma-chain specific-) peroxidase antibody (1:20,000, Sigma Aldrich) and colorized with 3,3′,5,5′-Tetramethylbenzidine (TMB) liquid substrate. Color change was induced with 1% HCl and the absorbance values were measured at 450 nm with VMax microplate reader (Molecular Devices).

2.7. Protein Characterization with SDS-PAGE and Western Blotting. The protein was characterized with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. Samples were reduced in a 1x sample sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting.

2.8. In-Solution Enzymatic Digestion. 100 μg of protein sample obtained from protein A affinity chromatography was denatured with 50 mM ammonium bicarbonate (ABC) and 2 M urea. The sample was reduced by 100 mM DTT for 1 h at 35°C. After incubation, the sample was alkylated with 100 mM IAA for 1 h in a darkroom and digested with trypsin (0.125 μg/μL) overnight at 37°C. Digested sample was dried in a SpeedVac and stored in −20°C or reconstituted in 1% FA for desalting. SPE micro-spin column (i.d. 180 μm, length 20 mm, and particle size 5 μm; Waters, USA) with an autosampler. The peptides were desalted in the trap column for 10 min at a 5 μL/min flow rate. The trapped peptides were back-flushed into a homemade C₁₈ trap column (i.d. 100 μm, length 200 mm, and particle size 3 μm) for separation. Mobile phases A and B were composed with 100% H₂O containing 0.1% FA and 100% ACN containing 0.1% FA, respectively. The LC gradient began at 5% mobile phase B and was maintained for 15 min. The gradient was ramped up to 15% B for 5 min, 50% B for 75 min, and 95% B for 1 min. 95% B was maintained for 13 min and decreased to 5% B for 1 min. The column was finally re-equilibrated with 5% B for 10 min. LTQ Orbitrap Elite mass spectrometer (Thermo Fisher Scientific) equipped with a nano-electrospray source was used to analyze the separated peptides. The electrospray voltage was set to 2.2 kV and the LTQ Orbitrap Elite mass spectrometer (Thermo Fisher Scientific) was operated in data-dependent mode during the chromatographic separation. The MS acquisition parameter for full-scan resolution was 60,000 in the Orbitrap for each sample. Data-dependent MS/MS scans were acquired by collision-induced dissociation (CID) and higher-energy collision dissociation (HCD). CID and HCD fragmentation scans were acquired in linear trap quadrupole (LTQ) mode with a 30-ms activation time and in Orbitrap at resolution 15,000 with a 20-ms activation time. A 35% normalized collision energy (NCE) and 5.0 Da isolation window were used for CID and HCD analyses. Previously fragmented ions were excluded for 300 s in all MS/MS scans. Raw mass data acquired were analyzed with the automated Glycoproteome Analyzer (GPA) algorithm to identify glycopeptides and then the resulting identified glycopeptides were further confirmed manually with careful investigation in retention time, chromatographic peak shape, isotopic mass distribution pattern, and fragmented ion matching.

2.9. In-Gel Enzymatic Digestion. In-gel protein sample excised from SDS-PAGE was repeatedly dehydrated with 500 μL ACN for 10 min at room temperature. The gel slices were reduced with 75 μL DTT for 30 min at 56°C, alkylated with IAA for 20 min in a darkroom, and destained with 500 μL 100 mM ammonium bicarbonate/acetonitrile (ABC/ACN) for 30 min with periodic vortexing. After dehydation, the gel slices were rehydrated with 10 mM ABC and 10% ACN buffer containing trypsin and digested at 37°C overnight. 5% FA/ACN (1:2, vol/vol) was added to the digested mixture and incubated at 37°C for 15 min with slight vortex. The digested sample was dried in a SpeedVac and either stored in −20°C or reconstituted in 0.1% FA for MS/MS analysis. Aliquot of the tryptic digests was applied to SPE micro-spin column, washed twice, and eluted twice with 30 μL elution buffer. The eluted peptide sample was dried in a SpeedVac.

2.10. LC-ESI-MS/MS Analysis. Tryptic peptide sample reconstituted with water containing 0.1% formic acid was separated by a Nano Acquity UPLC system (Waters, USA). 5 μL aliquot of the peptide solution was applied to a C₁₈ trap column (i.d. 180 μm, length 20 mm, and particle size 5 μm; Waters, USA) with an autosampler. The peptides were desalted in the trap column for 10 min at a 5 μL/min flow rate. The trapped peptides were back-flushed into a homemade C₁₈ trap column (i.d. 100 μm, length 200 mm, and particle size 3 μm) for separation. Mobile phases A and B were composed with 100% H₂O containing 0.1% FA and 100% ACN containing 0.1% FA, respectively. The LC gradient began at 5% mobile phase B and was maintained for 15 min. The gradient was ramped up to 15% B for 5 min, 50% B for 75 min, and 95% B for 1 min. 95% B was maintained for 13 min and decreased to 5% B for 1 min. The column was finally re-equilibrated with 5% B for 10 min. LTQ Orbitrap Elite mass spectrometer (Thermo Fisher Scientific) equipped with a nano-electrospray source was used to analyze the separated peptides. The electrospray voltage was set to 2.2 kV and the LTQ Orbitrap Elite mass spectrometer (Thermo Fisher Scientific) was operated in data-dependent mode during the chromatographic separation. The MS acquisition parameter for full-scan resolution was 60,000 in the Orbitrap for each sample. Data-dependent MS/MS scans were acquired by collision-induced dissociation (CID) and higher-energy collision dissociation (HCD). CID and HCD fragmentation scans were acquired in linear trap quadrupole (LTQ) mode with a 30-ms activation time and in Orbitrap at resolution 15,000 with a 20-ms activation time. A 35% normalized collision energy (NCE) and 5.0 Da isolation window were used for CID and HCD analyses. Previously fragmented ions were excluded for 300 s in all MS/MS scans. Raw mass data acquired were analyzed with the automated Glycoproteome Analyzer (GPA) algorithm to identify glycopeptides and then the resulting identified glycopeptides were further confirmed manually with careful investigation in retention time, chromatographic peak shape, isotopic mass distribution pattern, and fragmented ion matching.
Table 1: List of tryptic glycopeptides from triplicate analysis of reference rAb.

| No. | Glycopeptides | Ion Charge (+) | Detected Mass (m/z) | Run 1 | Run 2 | Run 3 |
|-----|---------------|----------------|--------------------|-------|-------|-------|
| 1   | EEQYNSTYR     | 2              | 1142.959           | 27.70 | 92380184 | 28.39 | 32709010 | 28.40 | 1039768 |
| 2   | EEQYNSTYR     | 2              | 1215.989           | 27.41 | 37213978 | 28.31 | 72807858 | 28.30 | 18879486 |
| 3   | EEQYNSTYR     | 2              | 1244.499           | 27.70 | 91063963 | 28.39 | 15496872 | 28.42 | 15407082 |
| 4   | EEQYNSTYR     | 2              | 1318.029           | 27.40 | 1244.499 | 27.70 | 91063963 | 28.39 | 15496872 |
| 5   | EEQYNSTYR     | 3              | 946.380            | 27.60 | 7447300  | 28.32 | 5456578  | 28.30 | 7199469  |
| 6   | EEQYNSTYR     | 3              | 913.355            | 28.28 | 188888   | 28.88 | 2653427  | 28.90 | 1300713  |
| 7   | EEQYNSTYR     | 3              | 1297.018           | 27.40 | 50607743 | 28.25 | 6312278  | 28.21 | 20746171 |
| 8   | EEQYNSTYR     | 3              | 1442.557           | 28.10 | 29760350 | 28.81 | 55607567 | 28.80 | 28356277 |
| 9   | EEQYNSTYR     | 3              | 1398.554           | 27.38 | 28576747 | 28.25 | 58136525 | 28.20 | 20561471 |
| 10  | EEQYNSTYR     | 3              | 1029.738           | 28.10 | 5127014  | 28.80 | 36633653 | 28.85 | 12664730 |
| 11  | EEQYNSTYR     | 3              | 1000.398           | 27.54 | 2426554  | 28.32 | 4640770  | 28.30 | 6085787  |
| 12  | EEQYNSTYR     | 3              | 1203.473           | 27.50 | 54231608 | 28.28 | 16939021 | 28.29 | 69462798 |
| 13  | EEQYNSTYR     | 3              | 986.723            | 27.36 | 3496656  | 28.10 | 2860860  | 28.10 | 10937706 |
| 14  | EEQYNSTYR     | 3              | 1083.755           | 28.10 | 8174096  | 28.81 | 18861846 | 28.80 | 25603836 |
| 15  | EEQYNSTYR     | 3              | 1110.808           | 28.52 | 1129042  | 29.20 | 3397473  | 29.29 | 1181060  |
| 16  | EEQYNSTYR     | 3              | 1284.497           | 27.40 | 10213292 | 28.18 | 733668   | 28.21 | 14024518 |
| 17  | EEQYNSTYR     | 3              | 973.049            | 27.36 | 4256849  | 28.18 | 638835   | 28.20 | 4721699  |
| 18  | EEQYNSTYR     | 3              | 1070.081           | 28.04 | 5703122  | 28.74 | 18933930 | 28.70 | 8336957  |
| 19  | EEQYNSTYR     | 3              | 1365.524           | 27.40 | 55356342 | 28.18 | 559700   | 28.21 | 5262829  |

*MS_Intensity is based on the summation of 3-top monoisotopic mass peak intensity [21]. If simultaneously detected, MS_Intensity values of 2+ and 3+ mass ions are summed.*

3. Results and Discussion

3.1. LC-ESI-MS/MS Analysis of Reference rAb Glycoprotein. The reference rAb glycoprotein desalted with a HiTrap Mabsselect SuRe protein A column (GE Healthcare) according to manufacturer’s protocols was quantified by UV spectrometry, ELISA assay, and BCA assay and characterized by SDS-PAGE and Western blotting. The reference rAb glycoprotein sample desalted was reduced, alkylated in a darkroom, and digested with trypsin overnight. The digested sample was desalted with a SPE micro-spin column and analyzed by LC-ESI-MS/MS coupled with CID and HCD fragmentation techniques.

Figure 1 shows a base peak chromatogram (BPC) obtained from the analysis of the tryptic digests of the reference rAb glycoprotein. The obtained raw mass data was analyzed with GPA algorithm and the automatically assigned glycopeptides were further validated manually. Among the identified glycopeptides, EEQYNSTYR_(3(hexose)_3(N-acetylhexosamine)_1(fucose)_0(sialic acid)) was eluted at the retention time (RT), 28.25 min. The extracted ion chromatogram of the monoisotopic peak pattern of the precursor ion (2+) for glycopeptide EEQYNSTYR_(3(hexose)_3(N-acetylhexosamine)_1(fucose)_0(sialic acid)) was illustrated in Figure 1. Tandem mass spectra of the glycopeptide EEQYNSTYR_(3(hexose)_3(N-acetylhexosamine)_1(fucose)_0(sialic acid)) were also shown in Figure 2. We confirmed that fragment ions were well-matched with their structures.

The analysis results of the reference rAb obtained by triplicate sample preparation and mass analyses were summarized in Table 1. LC-ESI-MS/MS with tryptic digestion detected 20 consistent glycosylation patterns with similar distributions. The glycosylation patterns normalized for each run were graphed together in Figure 3. We observed three major fucosylated glycopeptides, EEQYNSTYR_(3(hexose)_3(N-acetylhexosamine)_1(fucose)_0(sialic acid)) and EEQYNSTYR_(4(hexose)_4(N-acetylhexosamine)_1(fucose)_1(sialic acid)), which are reportedly predominant glycoforms found in recombinant antibodies produced in CHO cells [7, 24, 25]. In addition, high mannose forms with the relative ratios <13% were also observed—EEQYNSTYR_(2(hexose)_2(N-acetylhexosamine)_1(fucose)_0(sialic acid)) and EEQYNSTYR_(1(hexose)_1(N-acetylhexosamine)_1(fucose)_1(sialic acid)). These glycosylation patterns have been frequently observed in rAb...
3.2. Comparative Analysis of Glycosylation Patterns in Biosimilar rAbs. Samples of biosimilar rAbs were obtained from either transiently transfected cells or a stably transfected cell line and referred to as biosimilar rAb A and B, respectively, for clarification purposes. The purified biosimilar rAbs A and B were digested with trypsin and analyzed with LC-ESI-MS/MS. The obtained raw mass data was analyzed with GPA algorithm and the identified glycopeptides were further validated manually. The glycosylation patterns acquired for the biosimilar rAb A and B were individually normalized and the mapping results were compared with those obtained from the analysis for the reference rAb sample (see Figure 4).

The major glycoforms for the biosimilar rAb A and rAb B as well as the reference rAb were consistent—EEQYNSTYR\(_3\_3\_1\_0\), EEQYNSTYR\(_3\_4\_1\_0\), and EEQYNSTYR\(_4\_4\_1\_0\). However, we observed variations in the minor glycosylation species not only between the reference rAb and biosimilar rAbs but also between the two biosimilar rAbs samples produced using different methods. The biosimilar rAb A and B displayed a decreased abundance for EEQYNSTYR\(_3\_4\_1\_0\) and an increased abundance for EEQYNSTYR\(_4\_4\_1\_0\) with the addition of galactose, in comparison to the reference rAb. The biosimilar rAb B displayed higher abundance for glycans with sialic acids—EEQYNSTYR\(_4\_4\_1\_1\), EEQYNSTYR\(_5\_4\_1\_1\), and EEQYNSTYR\(_5\_4\_1\_2\) in comparison to the biosimilar rAb A. These results suggest that LC-tandem mass spectrometry can be a highly sensitive tool for distinguishing subtle differences in minor glycosylation species.

3.3. Glycosylation Patterns in Mutagenized rAb Glycoprotein. We tested the potential validity of using LC-tandem mass spectrometry in the determination process for the similarity of biosimilar products to the reference materials in biosimilar samples with a single mutation in amino acid sequences. Unintentional mutations that arise during protein production can cause changes in glycosylation patterns, which in turn can impact the safety, quality, and potency of the recombinant rAbs [28]. A tryptophan to valine amino acid mutation was created in the vicinity of the glycosylation site of Adalimumab to test its effect on the pattern of glycosylation to be detected using LC-tandem mass spectrometry. Interestingly, the single amino acid mutation caused the inability for biosimilar rAb to bind to the protein A affinity column (data not shown). So, instead of purifying the mutagenized rAb using protein A columns, we performed the analysis using in-gel samples. Unpurified samples were analyzed by SDS-PAGE and the expected heavy chain and light chain bands were observed at 51kDa and 26kDa, respectively (see Figure 5). The bands excised from the gel were subjected to in-gel
Figure 2: Tandem mass spectra of the glycopeptide EEQYNSTYR, obtained by (a) collision-induced dissociation and (b) higher-energy collision dissociation.
tryptic digestion. The digested sample was desalted by ZipTip for mass spectrometry.

Prior to analysis of the tryptic digests of the mutagenized rAb glycoprotein, a comparative analysis was performed between the in-solution reference rAb and the in-gel reference rAb glycoprotein to ensure the reliability and repeatability of the developed glycopeptide mapping methods (see Figure 6). The results in Figure 7 show that the mass
Figure 5: SDS-PAGE result for mutagenized rAb samples (lanes 1-3) and reference rAb glycoprotein (lane 4). Heavy chain and light chain bands were observed at 51 kDa and 26 kDa, respectively.

Figure 6: Comparative analysis for in-solution and in-gel rAb samples. In-solution sample is applied to a protein column and subject to tryptic digestion. In-gel sample is separated from SDS-PAGE gel and destained before tryptic digestion.

We then conducted the analysis of the in-gel tryptic digests of the mutagenized rAb glycoprotein and compared its pattern against the in-gel tryptic digests of the reference rAb, obtained, respectively, from the in-solution and the in-gel method. The two methods for sample preparation provided comparable results, implying that the in-gel method can be complimentary to the in-solution method for mapping protein glycosylation for our purposes.

Spectrometry analysis of both the in-solution and the in-gel reference rAb samples yielded similar results with consistent glycosylation patterns, proving the reliability of the method.

Overall, we observed a significant increase of sialylated, galactosylated, and branched glycoforms in the mutagenized rAb glycoprotein. These substantial differences in glycosylation patterns can be attributable to the single amino acid mutagenesis and are as expected from the observation that the single amino acid change also caused the rAb’s inability to bind to protein A column.
species can become the basis for the failure of the biosimilar rAb in proving similarity with the reference rAb within the scope of the FDA guideline. The possible variations in functionality and/or immunogenicity of the biosimilar rAb over the reference rAb can only be determined with additional tests following this report.

We also tested the effect of a single amino acid mutation on the overall pattern of glycosylation of the biosimilar rAb. Coincidently, this single amino acid substitution introduced within the CH2 domain of the heavy chain (at position C41 by IMGT codon numbering or 290 by Kabat numbering) of IgG molecule caused the inability of the mutagenized biosimilar rAb to bind to protein A column. Without a readily available method to purify the mutagenized biosimilar rAb, we used in-gel based analysis of the glycoproteins. The in-gel based method was first proven to be effective when compared with in-solution based method in the analyses performed using the reference rAb sample. The in-gel based analysis of the mutagenized rAb glycoprotein showed that a single amino acid mutation in the vicinity of the IgG Fc glycosylation site can cause an increased level of variations on the overall pattern of glycoforms and with more pronounced variations on the minor species. The fact that a single mutation in the amino acid sequence of biosimilar can cause a significant change in the resulting glycosylation patterns and/or physiochemical properties emphasizes the importance of detailed glycopeptide mapping methods, such as LC-tandem mass spectrometry-based method as described our experiment, which can distinguish even slight variations on the minor glycoform species as an added accuracy in biosimilar development.

### Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

### Conflicts of Interest

The authors declare that they have no conflicts of interest.

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