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Quantitative comparison of the N-glycosylation of therapeutic glycoproteins using the Glycosimilarity Index. A tutorial

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1. Introduction

Considering the increasing role of protein therapeutics in the pharmaceutical market, proven by the fact that currently most of the high revenue drugs are monoclonal antibodies, Fc-fusion proteins and glycoprotein based new modalities, their comprehensive characterization is of high importance [1]. In most therapeutic mAbs this characterization process means the analysis of the N-glycosylation present at the conserved Asn297 site located of the C_H2 domain in the Fc region of antibody based molecules. Generally, these N-glycans are deeply involved in the biological activity, physicochemical properties and serum half-life of a glycoproteins. Fc glycosylation in mAbs and Fc fusion proteins affect their effector functions such as antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC). Another important sugar residue dependent function of glycosylated biotherapeutics can be the anti-inflammatory effect influenced by terminal sialylation [2]. Clearance of mAbs are also affected by the type of glycosylation they carry. For instance, it has been shown that the so called G0 glycoform (asialo-, agalacto-, biantennary core fucosylated complex N-glycan, also referred to as FA2 by the Oxford nomenclature [3]), which contains no galactoses and therefore presents terminal N-acetylglucosamines (GlcNAc), is bound by a C-type lectin, a mannose receptor, thus, cleared by dendritic cells and macrophages [4,5]. Similarly, IgGs bearing high mannose-type glycans exhibit reduced serum half-life [6]. These findings have strong implications in therapeutic antibody quality control, suggesting that glycoforms should be carefully controlled, even at low levels if immunogenic such as the α1-3 Gal or N-Glycolylneuraminic acid epitopes. In most instances, the above listed functions and features are parts of the mode of drug action in relation to their safety and clearance profile [7]. Therefore, detailed analysis of these glycans provides important critical quality attribute (CQA) information.

Given its importance, regulatory guidelines require the characterization and monitoring of N-glycans since glycoproteins always display a heterogeneous set of glycans depending on the genetics of the host cell line as well as the upstream and downstream bioprocesses. Biotherapeutics are usually developed using the so called quality by design (QbD) approach, where glycosylation is often a very important part of the product design as it can enhance such molecular abilities as increased half-life, specificity and efficacy. Since glycosylation is not template driven, i.e., not coded in DNA, predicting glycosylation is very complex and difficult given the number of factors and cellular processes that must be considered to obtain the desired glycosylation profile. These factors include the expression levels of relevant glycosyltransferase/glycosidase enzymes, the level of monosaccharides and pseudo-sugars present during fermentation, as well as bioprocessing parameters, like temperature, pH, O_2 and CO_2 level, etc.

Thus, comprehensive characterization of all attached N-glycan structures including positional and linkage information is critical and should be monitored from early through late stage development to commercial manufacturing. WHO guidelines on biotherapeutics [8] and the International Conference on Harmonization (ICH) Q6B [9] mandate also stated that PTMs, such as glycosylation should be identified and adequately determined. Due to the significant impacts of the attached carbohydrate structures on safety and therapeutic efficacy as discussed above, analysis of the glycan profiles of therapeutic glycoproteins is an inseparable part of the quality control strategy for glycosylated biopharmaceuticals.

Since, glycosylation is considered to be critical for quality and it can be easily influenced by small manufacturing variations or changes, glycan related attributes must also be assessed during comparability testing. Consequently, it is also essential for biosimilars to quantitatively show their resemblance in terms of their glycosylation to the reference product [10].

Glycan analysis is a challenging task considering the possible high complexity of these molecules. Their analysis by liquid phase separation techniques such as liquid chromatography or capillary electrophoresis is hindered by the lack of chromophore/fluorophore moieties, requiring derivatization, in most cases with fluorescent tags for enhanced detection sensitivity and specificity. For capillary electrophoresis analysis, carbohydrates are labeled by a charged fluorophore to respectively support their electromigration and sensitive detectability [11]. Rapid exploratory and structural elucidation of the separated sugars is usually accomplished by applying the so called glucose unit (GU) value method, that practically normalizes the migration/elution time of the separated peaks to an oligosaccharide ladder [12]. The calculated GU values are then used to search relevant glycan databases (e.g., www.GlycoStore.org) and the structures of the individual carbohydrates are suggested/classified with adequate probability. To simplify the calculation process and increase precision, bracketing or the recently introduced triple internal standard based approach is applied [13]. However, for exact characterization of the separated gly-

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cans, exoglycosidase mediated carbohydrate sequencing and MS analysis are also important [14,15].

Biosimilars, by definition, are copy versions of already authorized biological medicinal products with demonstrated similarity in physicochemical characteristics, efficacy and safety, based on comprehensive analytical and clinical similarity studies [16]. Glycosimilarity represents a substantial subset of biosimilarity [17] and it reflects to similarity in terms of the glycosylation related critical quality attributes (gCQAs) especially macro- and micro-heterogeneity. For therapeutic monoclonal antibodies and Fc fusion proteins these attributes are usually as follows: afucosylation level (fucosylation affects ADCC function via FCγRIII binding), total terminal galactosylation (antennary galactosylation influences CDC activity), total sialylation (terminal sialylation enhances anti-inflammatory effects), as well as the amount of high mannose structures and glycan species having terminal GlcNAc (both for their potential effect on serum half-life). If present, the analysis and quantification of immunogenic glycan residues are also important parts of such study focusing on the detection and comparison of the levels of the following residues: alpha (1,3)galactose, beta (1,2)xylose, alpha (1,3)fucose and N-glycolylneuraminic acid (Neu5Gc).

2. The glycosimilarity concept

The approach and rules of establishing biosimilarity are defined by leading regulatory agencies, including but not limited to the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA) [18,19]. There are more and more biosimilar candidates have been granted approvals in recent years with the related documentation (briefing documents, papers with detailed analytical similarity data sets) publicly available about their analytical similarity approach and results. Therefore, the major requirements by the leading agencies nowadays are considered to be relatively clear across the industry. Both EMA and FDA recommends the use of a three-tier based approach for the criticality ranking of the different CQAs. Based on this ranking they suggest to use different acceptance criteria/quality ranges to the attributes considering their importance when assessing similarity between the reference medicinal product (RMP) and its biosimilar candidates. For Tier 1 or highly critical attributes the FDA suggests the use of equivalence testing, while EMA recommends the use of quality range (±1.5 SD) with a 95% confidence level. For Tier 2 ranked attributes a quality range method is suggested by both agencies that is created using a ± 3 SD upper and lower limit around the RMP mean also with a 95% confidence. In case of the least critical QAs (Tier 3) graphical or numerical comparison supposed to be sufficient without a predefined similarity range [18,19]. EMA has also been emphasizing the importance of testing the reference medicinal product and biosimilar candidate samples head-to-head in the same assay using different, preferably orthogonal analytical methods whenever feasible. Therefore, defining similarity, or more precisely the glycosimilarity, requires a well-defined scoring system and rules for comparative characterization such as the one introduced several decades ago by Altman and Bland for measurements in medicine [20]. Recently, their approach was further developed by Karlsson et al. [21] to be used for similarity comparisons of surface plasmon resonance sensorgrams to characterize and compare different protein interactions.

In this tutorial, an efficient and complex similarity scoring system is presented using a step-by-step approach to define numerical similarity between the N-glycan profile of a model therapeutic monoclonal antibody and an artificially generated spiked test N-glycan pool. The approach is based on the calculation and combination of two different similarity scores. The first one is the profile similarity score, which is calculated by comparing each and every single data points of the normalized electropherogram (or chromatogram) of the test item to a pre-defined quality range that is based on the manufacturing variability of the reference lot. The second score is the compositional similarity, which is based on comparing the levels of the different gCQA groups to the similarity range that is created just like in the previous case based on the compositional variations of the released N-glycan pool from 6 different manufacturing lots. The mean of these two scores results in the final Glycosimilarity Index (GI). One benefit of using such an index is in complex DoE studies, used for process development and process characterization activities or during clone selection, the Glycosimilarity Index can be used as a single response that describes the closeness of the N-glycosylation profile of the test item to the desired target or reference profile. Using lower number of responses allow the generation of more simplified, therefore, more accurate and reliable models that eventually lead better and more robust manufacturing processes. In addition, the use of the GI is also beneficial in comparability and similarity studies and allows a clear, simple and easy comparison between the two samples and therefore better decision making. Application of the GI in clone selection studies enables simple differentiation between the N-glycosylation profiles of the candidates, while in similarity or comparability studies it will allow easier comparison between the different manufacturing lots or the biosimilar candidate and its reference product.

3. Theory

Calculation of the similarity scores is based on the approaches of Altman and Karlsson [20,21]. Percentage scores are generated with a simple mathematical calculation for considering all data points (i.e., UV absorbance or fluorescence unit values) for the peaks of interest, falling inside and outside of the Tier pre-defined tolerance limits defined by ± SD of the mean value from the different reference manufacturing lots. Points located inside the limits, regardless of their distances from the mean are scored with 100% similarity as shown in Fig. 1 (first term in Equation (1)). On the other hand, the contribution of points falling outside of the tolerance limits are corrected by using a sum of squared distances to the mean (second term in Equation (1)). Consequently, the points outside the tolerance limits reduce the similarity score.

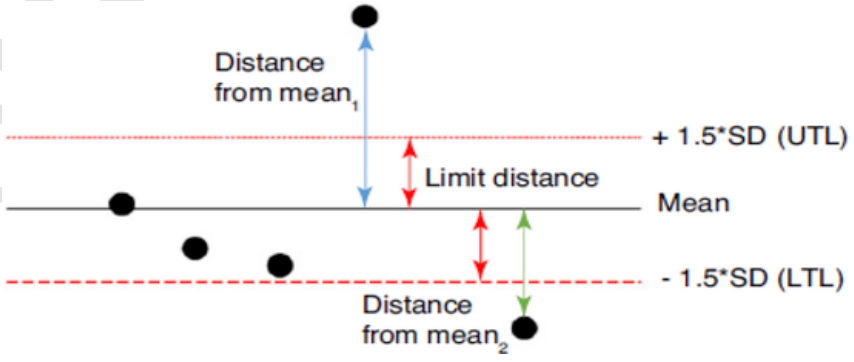

Fig. 1. The glycosimilarity scoring concept. UTL: upper tolerance limit, LTL lower tolerance limit.

$$A_i = Y_i + X_i \cdot \sum_{j=1}^{n} \frac{\left(\left(x_{uj} - x_{lj}\right) - \left(\overline{x_j}\right)\right)^2}{\left(x_{sj} - \overline{x_j}\right)^2} \quad (1)$$

where, A_i represents the percentage of similarity of attribute i (i = 1, …,n), Y_i and X_i represent the percentage of data points inside and outside of the tolerance limits of that particular attribute, respectively. The upper and lower tolerance limits of point j are represented by x_{uj} and x_{lj}, respectively. The mean of the jth reference material points is $\overline{x_j}$, while, x_{sj} is the jth sample point falling outside the tolerance limits [17] as shown in Fig. 1. The worked example below details all the calculation steps for easier understanding.

Linking of the obtained N-glycosylation profile to safety, clearance or efficacy of the product is usually done through the definition of glycosylation related CQAs (gCQAs). These attributes in most cases are defined as important subsets of the major N-glycan subtypes, like total afucosylation, total high mannosylation, total sialylation or total terminal galactosylation, etc. Values are assigned to these attributes by summing up the relative percentage of the amounts of those oligosaccharides having these terminal sugar residues.

Since the peak area ratio of the different glycans within a gCQA is also very important, the composition similarity of the released glycan pool has to be considered as a major attribute. For example, the composition of the total afucosylation of Fc attached N-glycans is highly critical in terms of the expected ADCC response. A test mAb with 10% total afucosylation that only comprises of high mannose type glycans will have significantly different ADCC activity than that of a mAb where the 10% of afucosylation is a result of mainly complex type N-glycans, like G0, G1, G1' or G2. The overall glycosylation similarity (Equation (2)), the Glycosimilarity Index (GI) is composed of the mean of the profile and compositional similarity scores (both are calculated by Equation (1)) and the profile similarity is always ranked as a very high criticality (Tier 1) attribute, i.e., on the third power.

$$\mathrm{GI} = \frac{e^{t_{ip}} \cdot A_{ip} + \sum_{i=1}^{n} e^{t_{ic}} \cdot A_{ic}}{e^{t_{ip}} + \sum_{i=1}^{n} e^{t_{ic}}} \quad (2)$$

Where n is the number of attributes, A_{ip} is the percent value of the profile similarity score and A_{ic} is the similarity score of attribute i. All similarity scores are computed by using Equation (1). t_i is the tier rank factor, which is 3 for high (Tier 1), 2 for moderate (Tier 2) and 1 for attributes with low criticality (Tier 3). Profile similarity is always a high criticality ($t_{ip} = 3$) attribute, i.e., $e^3 = 20.09$, while the tier factor of compositional similarity (t_{ic}) can range from 1 to 3, i.e., $e^1 = 2.71$, $e^2 = 7.39$ and $e^3 = 20.09$.

In complex molecules containing occupied N-glycosylation sites at different locations of the molecule such as the fusion protein etanercept with two conserved N-glycosylation sites in its Fc portion and 4 distinct sites on the TNFα receptor part, sub-indexes shall be generated for each site types with different criticality. Thus, the generation of specific sub-indexes is required when different type of N-glycosylation sites are presented with such relevance as surface exposed sites that has no effect on the primary mechanism of action and conserved Fc sites affecting effector functions or sites that related for half-life and stability. These glycosylation sites on different parts of the protein therapeutic normally differ in their glycan composition as well as in the criticality of their N-glycosylation related attributes such as total sialylation or total afucosylation. For example, in an Fc fusion protein with significant effector functions, e.g., ADCC, the afucosylation level is considered to be a high criticality attribute for the Fc linked oligosaccharides ($t_i = 3$). On the other hand, for sugars attached to the N-glycosylation sites outside of Fc, this attributes have only very low criticality ($t_i = 1$). Therefore, these sites first have to be grouped based on the primary effect of their N-glycosylation, and the different groups shall be studied separately. When it comes to the calculation of the glycosimilarity index, it is necessary to calculate different sub-indexes for each groups. In general terms, lower Glycosimilarity Index implies greater effect of the observed glycosylation differences and consequently weaker overall similarity of a biosimilar candidate.

In practice, the Glycosimilarity Index calculation workflow includes the following steps:

1) Determination of the target profile by characterizing the reference lots
2) Identification of the glycosylation related attributes and assessment of their criticality
3) Classification of gCQAs and definition of their tolerance limits
4) Compositional and profile similarity scoring
5) Calculation of the Glycosimilarity Index

This tutorial provides a step by step approach for calculating the Glycosimilarity Index to obtain a single percentage score that represents the level of similarity between the different N-glycosylation profiles and their effect on safety, efficacy and immunogenicity relative to a defined reference target profile, that is generated from multiple lots of the reference product.

4. Glycosimilarity index calculation: A worked example

The reference mAb used for this worked example was an isotype IgG1κ monoclonal antibody expressed in CHO cells with intended target neutralization (Class II) and plausible ADCC function as part of its mode of action (MoA). It had only one N-glycosylation site in each heavy chains of the Fc region of the molecule at Asn297. The N-glycolylneuraminic acid (NGNA) content of the product was <0.1% of the total sialylation.

Step 1) Determination of the target profile by characterizing the reference lots

The released and fluorophore labeled glycans from six different production lots of the commercially available reference therapeutic mAb were analyzed in triplicates by capillary electrophoresis with laser induced fluorescent detection (CE-LIF) in two different sequences in different days to obtain the batch-to-batch and analytical variation of both the electropherogram profile and glycan composition. A representative electropherogram is shown in Fig. 2 with 15 structures identified based on their GU values (abbreviated structural names are listed in Table 1, following the Oxford nomenclature as cited in www.glycostore.org). Glucose unit (GU) values were assigned for each data points of the electropherogram by the GUcal software (freely available at www.GUcal.hu) to accommodate profile similarity calculations. The batch-to-batch peak area variances of the test mAb N-glycan compositions are depicted in Table 1.

Step 2) Identification of N-glycosylation related attributes and assessment of their criticality

The type of the biologic and genetic makeup of the host cell line used in the production of a glycobiotherapeutic drug defines its N-glycosylation attributes and their potential variances. Table 2 lists the abundance of the most important N-linked carbohydrate residues the various cell lines generate by species. (+++) depicts high abundance, (++) the presence, while (+) low abundance and (±) both the presence and absence reported in the literature. Terminal sialylation is an important attribute for anti-inflammatory properties, however, the N-glycolylneuraminic acid (Neu5Gc) subtype is immunogenic. Similarly, α1,3-Galactosylation (α1,3-Gal) and the presence of β1,6-xylose and alpha α1,3-core fucose are also considered as immunogenic residues. The presence of oligomannose structures is reportedly responsible for rapid clearance of the mAb from serum, suggesting that these structures are exposed and bind to the mannose receptor expressed by macrophages and other phagocytic cells [6].

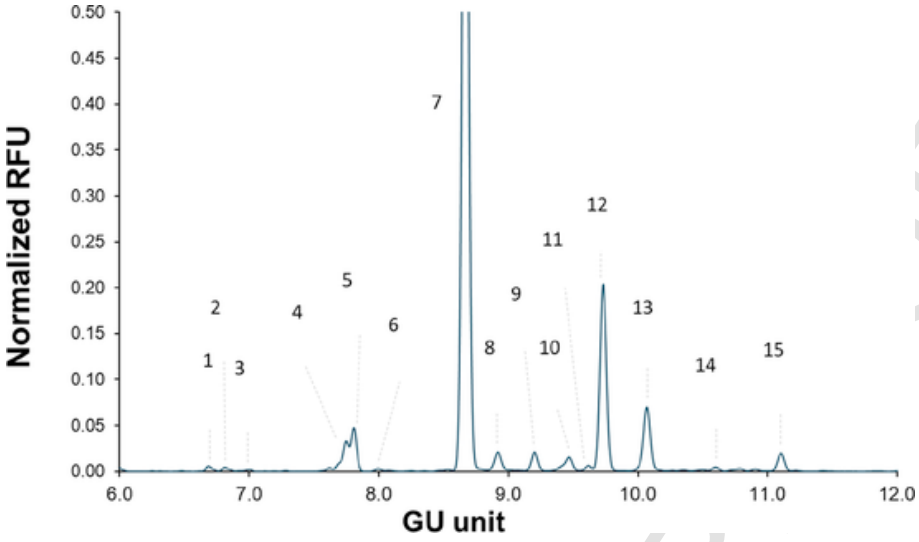

Fig. 2. N-Glycan profile of the test mAb. Structures of the numbered peaks are listed in Table 1. Conditions are given in the experimental section.

Table 1
Compositional batch-to-batch peak area variations of the test mAb analysis. The lower panel of the table depicts the percentage distribution of the major glycan subclasses of interest from glycosimilarity point of view.

Peak No.	Structure	Lot #1	Lot #2	Lot #3	Lot #4	Lot #5	Lot #6
		%	%	%	%	%	%
1	M3	0.4	0.4	0.3	0.3	0.3	0.4
2	FM3	0.6	0.6	0.3	0.4	0.4	0.3
3	FA1G1S1	0.5	0.5	0.3	0.3	0.3	0.2
4	A2	2.6	2.6	1.9	1.9	2.3	2.2
5	M5	3.5	3.5	2.3	2.3	3.2	2.8
6	FA2G2S1	0.6	0.6	0.3	0.3	0.3	0.2
7	FA2	62.5	62.5	62.4	61.5	66.1	67.3
8	A2[6]G1	2.0	2.0	1.6	1.7	1.8	1.7
9	FA1[6]G1	1.9	1.9	1.3	1.4	1.4	1.5
10	M7	1.8	1.8	1.4	1.5	1.7	1.6
11	FA3	0.5	0.5	0.3	0.3	0.4	0.4
12	FA2[6]G1	14.7	14.7	18.0	18.3	14.2	14.3
13	FA2[3]G1	6.0	6.0	6.8	7.0	5.5	5.4
14	M8	0.5	0.5	0.4	0.4	0.5	0.3
15	FA2G2	1.8	1.8	2.4	2.5	1.6	1.5
	Σ	100	100	100	100	100	100
	Terminal Gal %	26.4	26.4	30.0	30.8	24.5	24.3
	Afucosylated %	11.0	11.0	8.0	8.1	9.8	9.0
	High mannose%	5.9	5.9	4.3	4.2	5.4	4.7
	Sialylation%	1.1	1.1	0.6	0.6	0.6	0.4
	Terminal GlcNAc %	65.6	65.6	64.6	63.7	68.8	69.9

Table 2
Abundances of N-glycosylation related attributes in different species [22].

N-Glycan attribute	CHO	BHK	NSO, SP2/0	Human	Yeast
Sialylation	+ +	+	+ + +	+ +	–
α2,6-sialyl	–	–	+	+	–
Neu5Gc	+	+ + +	+ + +	+/–*	–
α1,3-Gal	–	+	+ +	–	–
bisect. GlcNAc	–	–	–	+	–
α1,6-core Fuc	+	+	+ +	+	–
α1,3-core Fuc	–	–	–	–	–
β1,6-xylose	–	–	–	–	–
High-mannose	+	+	+	+	+ + +

CHO: Chinese hamster ovary; BHK: baby hamster kidney; NS0: nonsecreting murine myeloma; SP2: mouse hybridoma cells. + + + abundant presence + + presence + low presence – not present ± both, presence and absence reported, *possible presence of Neu5Gc from exogenous sources.

Table 3
Effects of Fc glycosylation on mAb product quality (PK: pharmacokinetics; PD: pharmacodynamics [23,24].

N-Glycan attribute	Safety/Immunogenicity	Biological activity	Clearance (PK/PD)
Term. Galactose	n.d.	+	-
Term. GlcNAc	n.d.	-	-
α1,3-Gal	–	n.d.	n.d.
Core Fucose	n.d.	+ +	n.d.
bisect. GlcNAc	n.d.	+	n.d.
High-mannose	n.d.	+	–
Neu5Gc	–	(–)	+
Sialylation	n.d.	(–)	+
α1,3-core Fuc	–	n.d.	n.d.
β1,6-xylose	–	n.d.	n.d.

+ positive impact; + + high positive impact; - negative impact; -- high negative impact; (±) potential impact.

The effects of critical glycan epitope related attributes should be evaluated based on safety/immunogenicity, biological activity/efficacy and clearance considerations. Table 3 shows the impact level of the various mAb sugar residues in view of their criticality. For example, the α1,3-galactose is highly immunogenic, so (--) reflects its highly negative impact. The core fucose on the other hand is critical for the biological activity/efficacy via ADCC function, thus has high impact (+ +). The criticality of some sugar epitopes are not yet determined or less important reflected by the n.d. (not determined) assignment. Once the criticalities of all CQAs are defined, their tolerance window should be defined.

Step 3) Classification of gCQAs and definition of their tolerance limits

In this exercise, a three Tier based criticality assessment of quality attributes were used. Classification of the different glycosylation related attributes were based on the abundance and effect of the certain gly-

can types to pre-defined mechanisms of action and on their contribution to the safety and clearance profile of the reference mAb. Tier 1 (high) includes gCQAs that are directly impacting the potency of the product, in this instance the ADCC function (level of core fucosylation) and clearance (high mannose structures). This required to fall within the mean ± 1.5 SD of the multiple reference product batches tested. Attributes ranked as Tier 2 has moderate impact on product quality, so the tolerance limits for these attributes are calculated as mean ± 3.0 SD. Finally, glycan types with low or no significant impact at these abundance levels are controlled by fix upper limits at the compositional level and mean ± 3.0 SD tolerance limit for profile similarity calculation.

Based on the three Tier information, the tolerance limits used for the critical sugar residues for the test mAb are shown in Table 4. As one can observe, the N-glycan attribute criticality of the total afucosylated (i.e., non-core fucosylated) structures are high, thus have ±1.5 SD tolerance limit for both profile and compositional similarity. It is important to note that total afucosylation also includes other structures without core fucose, e.g., high mannose structures. Actually, in profile similarity assessment the ±1.5 SD tolerance limit should apply for the high mannose structure subset anyway, considering its important role in clearance.

First, the relative fluorescence intensities (RFU) of the peaks were normalized based on the highest peak in the electropherogram to minimize the effects of analytical variability on the calculated similarity score. GU ranges used in similarity calculation were defined based on peak integration and the ranges were set according to peak start/end points. The glycosylation related N-glycan attributes, their criticalities and the corresponding tolerance limits for the test mAb are listed in Table 5. In case a particular glycan could be considered in two different attribute groups with different criticality, the stricter limits were applied during score calculation. For example, for the total amount of afucosylated structures the sum of structures without core fucose included high mannose structures. Therefore, in profile similarity assessment the ±1.5 SD tolerance limit was applied for high mannose structures too for this subset. The Mean as well as the Upper and Lower Tolerance limits were calculated using the normalized RFUs of the 6 different reference lots, point-by-point. Data points in the electropherograms outside of the assessed GU ranges (i.e., baseline) were considered in the similarity score calculation with a ±6.0 SD tolerance limit.

Step 4) Compositional and profile similarity scoring

To better demonstrate the Glycosimilarity Index calculation workflow, two artificial N-glycan profiles were generated by spiking the released and APTS labeled test mAb N-glycan pool with 1 and 2 pmol of both APTS labeled Man5 and FA2G2 glycan standards, referred to as Model 1 and Model 2, respectively. These models represent increase in the amounts of core fucosylated and high mannose structures, i.e., change the amounts of these two gCQAs. Fig. 3, panel A shows the superimposed CE-LIF traces of the mean profile of the six mAb lots with the spiked glycan pools with 1 and 2 pmol of Man5 and FA2G2 for Model 1 and Model 2, respectively. Panel B depicts the blown-up part of peaks 8–10. As one can observe in Panel A, the sizes of those two spiked peaks increased, while the area of the rest of the peaks remained practically unchanged. More importantly, the added amounts of these two glycans increased the corresponding peak sizes beyond the upper tolerance limits, consequently resulting in decreased glycosimilarity.

Table 4
Glycosylation related critical quality attributes (gCQA) and their tolerance limits.

gCQA	N-glycan Attribute criticality	Tolerance limit for profile similarity	Tolerance limit for compositional similarity
Terminal Gal %	Moderate (Tier 2)	±3 SD	±3 SD
Total afucosylated %	High (Tier 1)	±1.5 SD	±1.5 SD
High mannose %	High (Tier 1)	±1.5 SD	±1.5 SD
Sialylation %	Low (Tier 3)	±3 SD	<3%
Terminal GlcNAc %	Low (Tier 3)	±3 SD	±3 SD

Table 5
GU ranges of the individual structures with their tolerance limits.

Peak No.	Structure	GU range of peaks	Tolerance limit
1	M3	6.31–6.75	±1.5 SD
2	FM3	6.76–6.89	±3.0 SD
3	FA1G1S1	6.92–7.04	±3.0 SD
4	A2 (G0)	7.68–7.77	±1.5 SD
5	M5	7.77–7.85	±1.5 SD
6	FA2G2S1	7.95–8.44	±3.0 SD
7	FA2 (G0F)	8.58–8.75	±3.0 SD
8	A2 [6]G1	8.84–8.99	±1.5 SD
9	FA1 [6]G1	9.13–9.28	±3.0 SD
10	M7	9.32–9.53	±1.5 SD
11	FA3	9.57–9.64	±3.0 SD
12	FA2 [6]G1 (G1F)	9.65–9.82	±3.0 SD
13	FA2 [3]G1 (G1'F)	9.95–10.17	±3.0 SD
14	M8	10.55–10.66	±1.5 SD
15	FA2G2 (G2F)	11.03–11.18	±3.0 SD

Compositional similarity scores were calculated by Equation (1) using the concept delineated in Fig. 1. Table 6 depicts the N-glycosylation attributes of the major glycan subtypes of Terminal Gal %, Total afucosylated %, High mannose %, Sialylation % and Terminal GlcNAc % for the mean of the 6 lots, as well as for the two Model mixtures. The compositions were calculated for Model 1 spiked by 1 pmol and Model 2 spiked by 2 pmols of FA2G2 and Man5, each. As an example, the limit distance for the Tier 1 gCQA total afucosylated structures (second line in Table 6) with the mean value of 9.5 were LD = 2.0, thus, the lower tolerance limit (LTL) was 9.5–2 = 7.5; while the upper tolerance limit (UTL) was 9.5 + 2 = 11.5. After spiking with 1 pmol Man5 and FA2G2, the mean increased from 9.5 to 14.5, with the corresponding differences of 5 units. Considering the 2-unit limit distance (LD), the compositional similarity (CS) % score for this attribute was calculated based on Equation (1) as follows: 100 x ($2^2/5^2$) = 16.3, where 2 is the limit distance and 5 is the actual distance from the mean. The compositional similarity scores for the four other attributes were calculated similarly, considering 100% when the data points fell within the LTL and UTL and using the calculation scheme above for the other ones when the data points fell outside of the LTL and UTL. For the rest of the subtypes, this calculation resulted in 100% for Terminal Gal%, 23.8% for High Mannose, 100% for Sialylation and 86.7% for Terminal GlcNAc N-glycosylation attributes.

In profile similarity calculation, the three small peaks highlighted by the gray box in Fig. 3 (in the GU range of 8.58–9.65) were first assessed among the traces. Panel B in Fig. 3 depicts the blown up superimposed traces of the separation of the 6 test mAb lots for the individual peaks of 8, 9 and 10 with the median (dotted line) and the upper and lower tolerance limits (dashed lines) as defined in Table 5. The corresponding structures for these peaks were 8: A2 [6]G1 (terminal galactosylated afucosylated), 9: FA1 [6]G1 (terminal galactosylated fucosylated) and 10: M7 (high mannose) structures, all within the predefined tolerance limits of ±1.5, ±3.0 and ± 1.5, respectively. Therefore, considering each and every single data points of these peaks, based on the scheme of Fig. 1, all fell within the predefined tolerance limits. The corresponding calculation by Equation (1) resulted in

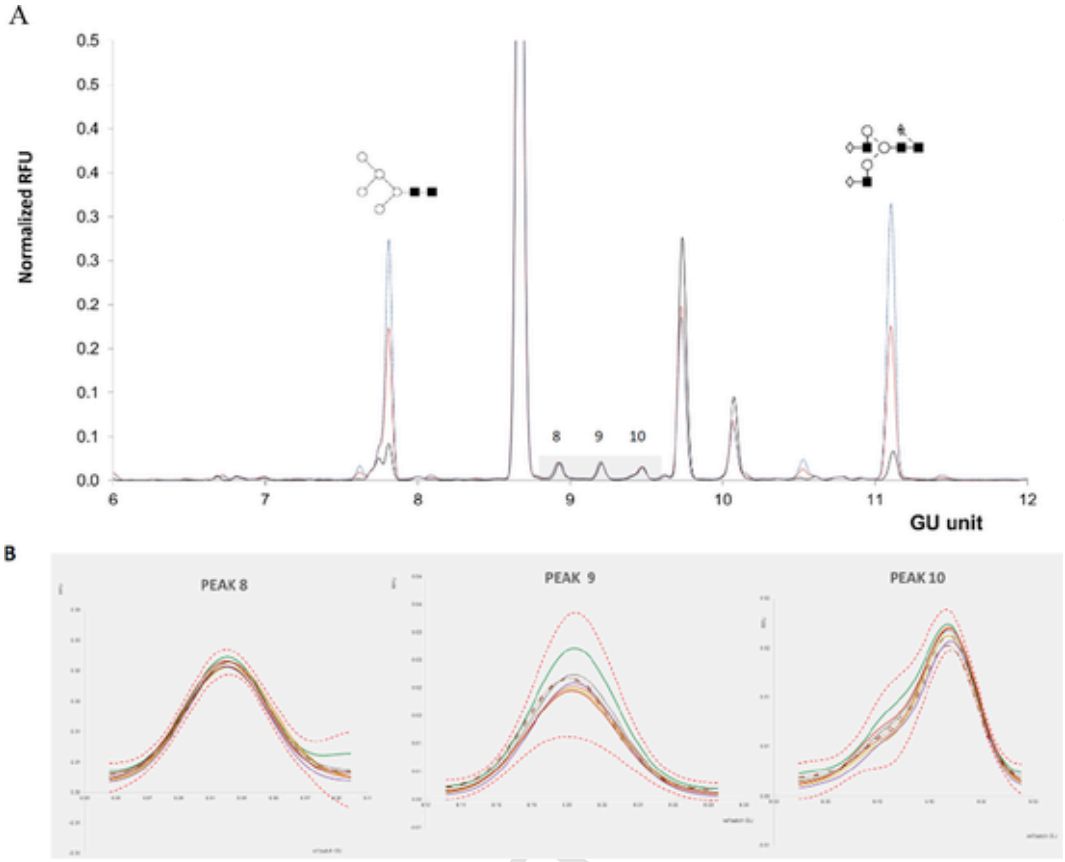

Fig. 3. Superimposed electropherograms (panel A) of the released and APTS labeled six mAb lots (black trace) with the spiked glycan pools containing 1 and 2 pmol of M5 and FA2G2 for Model 1 (red trace) and Model 2 (blue trace), respectively. Panel B depicts the blown-up part of peaks 8–10 with the black dotted and the red dotted lines representing the mean as well as the upper and lower tolerance limits, respectively. The other color traces correspond to the 6 production batches analyzed in the study.

Table 6
Calculated compositional Similarity (CS) score values.

N-glycosylation attributes	Mean	SD	LTL	UTL	LD	Model 1	CS% score Model 1	Model 2	CS% score Model 2
Terminal Gal %	27.1	2.7	18.8	35.3	8.1 (3SD)	30.1	100	33.5	100
Total afucosylated %	9.5	1.3	7.5	11.5	2.0 (1.5SD)	14.5	16.3	17.2	6.8
High mannose %	5	0.8	2.7	7.4	1.2 (1.5SD)	9.9	23.8	13.1	8.7
Sialylation %	0.7	0.3	0	1.6	0.9 (3SD)	0.7	100	0.6	100
Terminal GlcNAc %	66.4	2.5	59	73.7	7.5 (3SD)	58.5	86.7	52.1	26.8

100% profile similarity scores for these peaks, as the first term was 100% (all data points were inside the tolerance limits) and the second term was zero (no data points were outside of the tolerance limits). The same was true for all other features within the tolerance limits, i.e., for peaks 1–4, 6,7 and 11–14. Profile similarities were calculated for each and every single data points the same way for the spiked peaks of 5 (Man 5) and 15 (FA2G2). As one can see in Fig. 3 the Man 5 (peak 5) and FA2G2 (peak 15) peaks of both spiked traces exceeded the upper tolerance limit, thus, the second term of Equation (1) kicked in and decreased the profile similarity scores for Model 1 and Model 2, to 84.2% and 80.2% respectively.

Step 5) Calculation of the Glycosimilarity Index

The Glycosimilarity Index was then calculated by using Equation (2), taking in account of the profile and compositional similarity scores calculated above. The corresponding numbers for Worked Example Model 1 in Equation (2) were as follows:

$$GI_1 = \frac{e^{t_{ip}} \cdot A_{ip} + \sum_{i=1}^{n} e^{t_{ic}} \cdot A_{ic}}{e^{t_{ip}} + \sum_{i=1}^{n} e^{t_{ic}}}$$

$$= \frac{20.09 \cdot 84.2 + (7.39 \cdot 100 + 20.09 \cdot 16.3 + 20.09 \cdot 23.8 + 2.71 \cdot 100 +}{20.09 + (7.39 + 20.09 + 20.09 + 2.71 + 2.71)}$$

$$= 51.21\%$$

where $e^{t_{ip}} = 2.71^3 = 20.09$, A_{ip} is the percent value of the profile similarity score (84.2), and A_{ic} is the similarity score of the predefined attributes of Terminal Gal % (e^2 x 100%), Total afucosylated %, (e^3 x 16.3%) High mannose % (e^2 x 23.8%), Sialylation % (e^1 x 100%) and Terminal GlcNAc % (e^1 x 86.7%). All similarity scores were computed by Equation (1). Similar calculation for Model 2 resulted in $GI_{Model\ 2} = 41.12\%$.

5. Discussion

The Glycosimilarity Index gives a percentage score on the similarity of the N-glycosylation patterns in comparability and/or biosimilar-

ity studies of glycosylated therapeutic proteins. The use of this approach makes comparisons easily quantifiable in manufacturing between pre- and post-change batches or between the innovator and their biosimilar counterparts. This type of similarity scoring can also be used in system suitability testing when criteria such as "comparable/similar to reference standard" is used, allowing the specification of exact and objective numerical limits instead of subjective comparisons by the analysts. Using profile similarity scoring only can be a good approach to assess system suitability in N-glycan profiling assays by calculating and comparing exact values when assessing the comparable/similar to reference standard criteria. Please note that the type of the biologic and genetic makeup of the host cell line as well as the developed manufacturing process defines the N-glycosylation attributes and their potential variances. In the Worked Example of this study, only well-defined and characterized profiles were compared with relatively small variances for easier demonstration of the workflow. Based on the calculated compositional and profile similarity scores, the Glycosimilarity Index for Models 1 and 2 were 51.21% and 41.12%, respectively, both falling well below 80% [17], thus, exhibiting weak similarity in their N-linked glycan profiles, suggesting that the generated models would have significant differences in their Fc mediated functions and/or their contribution to clearance.

The Glycosimilarity Index (GI) also holds the potential to simplify and facilitate decision making for the biopharmaceutical industry during clone selection or process development studies representing all glycosylation profile changes with a single score. This makes the application of the Glycosimilarity Index extremely beneficial in design of experiment (DoE) models, where glycosylation changes can be defined as a single outcome only. This simplifies the models without the loss of important information and allows to create more robust and reduced dimensional design spaces.

6. Experimental

6.1. Chemicals and reagents

The Fast Glycan Sample Preparation and Analysis kit was from Sciex (Brea, CA). APTS labeled oligomannose 5 (Man5) and asialo-, galactosylated biantennary complex N-glycan, core-substituted with fucose (FA2G2) were from Prozyme (Hayward, CA). The PNGase F enzyme was from Asparia Glycomics (San Sebastian, Spain). The sodium cyanoborohydride (1 M, in THF) and all other chemicals were obtained from Sigma-Aldrich (St Louis, MO).

6.2. Reference mAb and sample preparation

Six batches of the test monoclonal antibody (Isotype IgG1κ) expressed in CHO cells with the intended mode of action of Class II (target neutralization) with plausible ADCC function were subject to glycosimilarity scoring in the Worked Example. The test mAb had only one N-glycosylation site at the conserved Asn297 on the Fc region of the molecule in both of the heavy chains. The N-glycolylneuraminic acid (NGNA) content of the product was $<0.1\%$ of total sialylation. Sample preparation followed the protocol of the Fast Glycan Analysis kit of Sciex. Models 1 and 2 were spiked pools of the 6 batches by the addition of 1 and 2 pmol of both Man5 and FA2G2 glycans, respectively. In all experiments the Fast Glycan Sample Preparation and Analysis kit was used with the respective reagent/buffer compositions and CE separation parameters.

6.3. CE-LIF analysis

In all separation experiments a PA 800 Plus Pharmaceutical Analysis System (Sciex) with laser induced fluorescent detection (λ_{ex} = 488 nm / λ_{em} = 520 nm) was used with 50 μm internal diameter (365 μm outside diameter) bare fused-silica capillary column (effective length: 50 cm, total length: 60 cm). The applied electric field strength was 500 V/cm in reversed polarity mode (cathode at the injection side) at 30 °C separation temperature. A water pre-injection by 3.0 psi for 5.0 s was followed by the injection of the sample (2.0 kV for 2.0 s) and the bracketing standard mixture of maltose and maltopentadecaose (1.0 kV for 1.0 s). For data acquisition and processing, the 32 Karat software, version 10.1 (Sciex) was used. Glucose unit values were determined by the freely available GUcal software (www.GUcal.hu).

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