Capillary electrophoresis in the N-glycosylation analysis of biopharmaceuticals

András Guttman

In N-glycosylation analysis of biopharmaceuticals, analytical need depends on the phase of the manufacturing process. All important glycoanalysis steps are thoroughly discussed. Carbohydrate sequencing by exoglycosidase arrays ~~isomers~~ is described in conjunction with capillary electrophoresis (CE) to identify linkage and positional. A possible automated workflow for ~~glyco-~~ analysis based on CE is outlined.

Keywords: Automated workflow; Biopharmaceuticals; Biotherapeutics; Capillary electrophoresis (CE); Carbohydrate sequencing; Glycoanalysis; High-sensitivity detection; Immunogenic epitope; N-glycosylation; Purity assessment

Abbreviations: ADCC, Antibody-dependent cellular cytotoxicity; APTS, 8-Aminopyrene-1,3,6-trisulphonic acid; CDC, Complement-dependent cytotoxicity; CE-LIF, Capillary electrophoresis-laser-induced fluorescence; CID, Collision-induced dissociation; ESI-MS, Electrospray ionization mass spectrometry; ETD, Electron-transfer dissociation; Gal, Galactose; Glc, Glucose; GlcNAc, N-acetylglucosamine; GU, Glucose unit; Hex, Hexose; HexNAc, N-acetylhexosamine; HILIC, Hydrophilic interaction chromatography; IMAC, Immobilized metal affinity chromatography; MALDI-MS, Matrix-assisted laser desorption/ionization mass spectrometry; Man, Mannose; NANA or Neu5Ac, N-acetylneuraminic acid; NGNA or Neu5Gc, N-glycolylneuraminic acid; PNGase-F, Peptide-N4-(N-acetyl-β-glucosaminyl)asparagine amidase; PTM, Post-translational modification; QBD, Quality by Design; RPLC, Reversed-phase liquid chromatography; UPLC, Ultrahigh-pressure liquid chromatography; WAX, Weak anion exchange

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1. Introduction

This article reviews the application of capillary electrophoresis (CE) and associated techniques for comprehensive N-glycosylation analysis of biotherapeutics. Progress in the field has been slow because the formation of glycans is not template driven, resulting in hundreds to thousands of possible structures. Site specificity and microheterogeneity makes their characterization even more challenging. With respect to fulfilling recent regulatory agency requirements, CE is considered to be a purely orthogonal method to liquid chromatography (LC) and mass spectrometry (MS), thus offering an important addition to the existing bioanalytical toolsets.

With the rapidly growing global biotherapeutics market [1], sensitive, high-resolution bioanalytical techniques are required for the comprehensive characterization of ~~biotherapeutics~~, including analysis of post-translational modifications (PTMs), higher order structures and protein aggregation, which are all important in understanding their behavior [2] (e.g., changes in PTMs may influence higher order structure formation, leading to possible malfunctioning of the biotherapeutic agent). One of the most prevalent PTMs is the carbohydrate moiety attached to the protein, which is closely monitored during the manufacturing process (i.e. clone selection, product development and lot release). Glycosylation is a highly dynamic PTM [3] and one can even extend the well-known central dogma with the glycosylation modification step as DNA → RNA → Polypeptide → Glycoprotein. Glycans protect proteins, orient binding faces, prevent non-specific interactions and increase protein stability, just to mention some of their most important roles (e.g., N-glycan shield large areas of protein surfaces from proteases).

Protein-glycosylation analysis, a sub-set of analytical glycobiology, provides information about the glycan structures that

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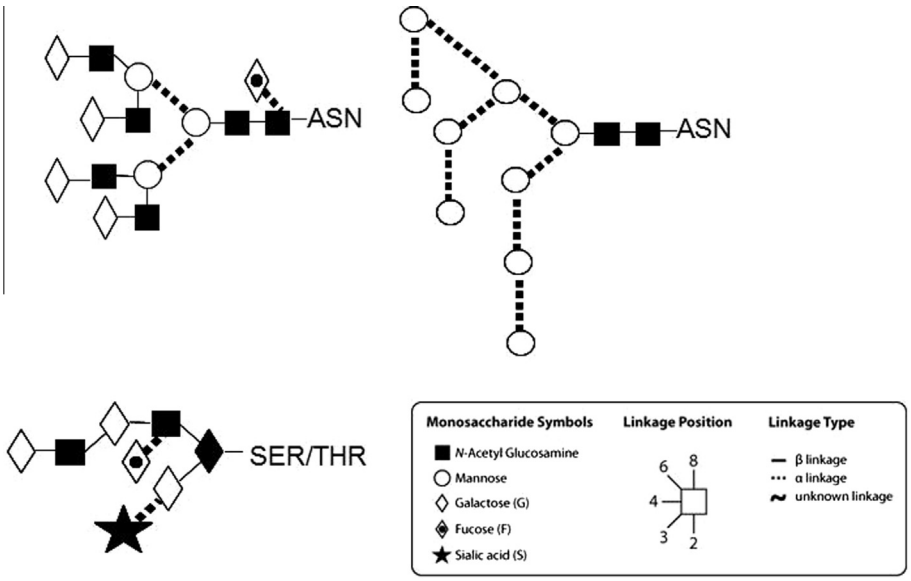

Figure 1. Main glycosylation types on biotherapeutics. Upper left: N-linked complex; Upper right: N-linked high mannose; Lower panel: O-linked structures. The symbolic representation of glycan structures was adapted from [54].

given cell types or organisms attach to biopharmaceuticals during production. The two major glycosylation types occur in biotherapeutics are N-linked and O-linked carbohydrates (see examples in Fig. 1). N-linked glycans are attached to the protein backbone ~~through~~ a well-defined consensus sequence of Asn-X-Ser/Thr (where X can be any amino acid except proline) through the side-chain amine group of the asparagine residue [4]. First, a 14-sugar precursor is co-translationally added to the asparagine in the polypeptide chain of the target protein. The structure of this precursor is common to most eukaryotes, and contains 3 glucose, 9 mannose, and 2 N-acetylglucosamine residues [3]. A complex set of reactions attaches this branched structure to a carrier molecule (dolichol), which is then transferred to the appropriate point on the polypeptide chain as it is translocated into the ER lumen and processed post-translationally. O-glycans on the other hand are bound through the side-chain OH group of serine or threonine residues with no consensus sequence requirement, and have at least 8 different core types [3].

Protein glycosylation involves the interplay of several hundred enzymes, and mutations in glycosylation processing enzymes can significantly alter the resulting sugar structures. In addition, glycosylation is specific to cells, proteins and sites, responsive to cell-culture conditions and may be modulated by bioprocessing conditions. As such, alterations in the process may even result in the incorporation of immunogenic epitopes (e.g., galactose-α-1,3-galactose (α-1,3-Gal) and N-glycolylneuraminic acid (NGNA) [5]~~}~~ ~~{e.g.,~~ Chinese hamster ovary (CHO) cells, a commonly used host cell line in the production of biotherapeutics, can synthesize galactose-α-1,3-galactose epitopes, as recent studies revealed [6]~~}~~. Therefore, in addition to the thorough glycosylation-profile analysis during the production of biotherapeutics, identification of potentially immunogenic epitopes is also very important and strongly advised by regulatory agencies [7,8].

2. Glycosylation-analysis options

Various tools are available for the analysis of carbohydrates. One of the most important is NMR [9], but the amount of material this method requires is often in the high-μg range [10]. Lectins, specific for particular glycosylation structures, are also widely used, ~~often~~ in an array format with or without antibodies (Abs) [11]. An important development was the introduction of hydrophilic interaction LC (HILIC) of fluorophore-labeled glycans [12]. This method, when in conjunction with exoglycosidase-array digestion, has been automated using a microtiter-plate-based system for ~~analysis at a low level of detection~~ [13]. MS is also a widely used technique in carbohydrate analysis [14], but, while powerful, it may not be quantitative [15]. In one approach, the released glycans are permethylated in strong base solution or using a sodium hydroxide microcolumn [16]. The permethylated derivatives have sufficiently different hydrophobicities to accommodate their separation by reversed-phase LC (RPLC), followed by matrix-assisted laser desorption/ionization MS (MALDI-MS) [17], or electrospray ionization MS (ESI-MS) [18]. ESI-MS is applied directly or after LC separation on porous graphitized carbon columns [19] or HILIC

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columns [20]. Albeit, this method can be sensitive, interpretation of MS/MS spectra may be difficult, and recovery from the porous graphitized carbon stationary phase maybe problematic, especially for highly sialylated structures [21]. Please note that the single-stage MS mode only reveals glycan composition (i.e. the number of Hex, HexNAc, ~~and~~ Neu5Ac), and gives no information on linkages and positions. However, the MS/MS mode can utilize fragmentation types (e.g., CID, ETD, and photodissociation) to provide some linkage and positional information. But, please note that labile residues can break off during the ionization process leading to false information that can be misleading during the development of the biopharmaceutical-manufacturing process.

Close attention should be paid to sialic acid and core fucosylation residues, which are especially sensitive during ESI-MS analysis. Loss of such residues of up to 50% was reported during ESI-MS analysis in comparison to liquid-phase analytical methods (e.g., HPLC) [22]. Glycan structures can also be somewhat assessed by LC/ESI-MS analysis of glycopeptides [23]. Another high-performance bioanalytical method for glycan analysis is CE with laser-induced fluorescent (LIF) detection [24]. CE-LIF can readily distinguish both linkage and positional isomers, so it became widely used for glycan analysis in the biomedical and biotechnology fields [25], especially in conjunction with ~~the~~ exoglycosidase-digestion array [26].

CE can be coupled to MS for the analysis of carbohydrates and glycopeptides for glycoform profiling of biotherapeutics [5,27,28]. Another advantage of CE-based systems is the option of easy multiplexing even up to 48 or 96 capillaries for high-throughput applications [29,30].

3. Capillary electrophoresis of sugars

For decades, CE has been extensively used for the analysis of fluorophore-labeled oligosaccharides in free solution [31], or gel-filled columns [32]. In both cases, the separation of the labeled sugar structures is based on differences in their hydrodynamic volume-to-charge ratio. In gel-filled capillaries, some interaction of the analyte molecules with the sieving matrix cannot be ruled out, as was suggested earlier [33]. To obtain CE migration-time data with high precision (<0.05% RSD), co-injection of a lower bracketing standard (migrating faster than any structures in the sample mixture) and a higher bracketing standard (migrating slower than any structures in the sample mixture) is highly recommended. Once the migration times are normalized by the bracketing standards, the corresponding glucose-unit (GU) values can be calculated using Equation (1) and used for database search for possible matching structures [34]:

$$GU_x = G_n + \frac{MT'_x - MT_n}{MT_{n+1} - MT_n} \quad (1)$$

where GU_x is the GU of the unknown glycan; G_n is the degree of polymerization of the preceding homooligomer; MT'_x is the corrected migration time of the unknown glycan; MT_n is the migration time of the preceding homooligomer; and, MT_{n+1} is the migration time of the subsequent homooligomer.

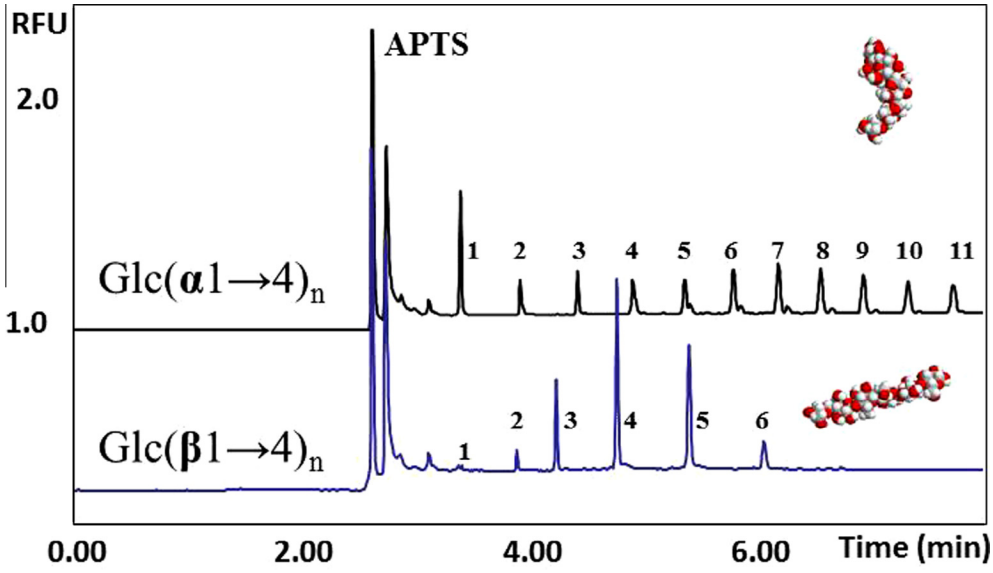

Figure 2. Comparative separation of APTS-labeled Glc(α1 → 4)$_n$ and Glc(β1 → 4)$_n$ ladders. The insets show the helical (Glcα1 → 4 linkage) and linear (Glcβ1 → 4 linkage) shapes of the respective sugar oligomers.

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Table 1. Calculated glucose unit values of Glc(β1 → 4)$_n$ homooligomers (second column) based on the degree of polymerization of Glc(α1 → 4)$_n$ oligomers (first column)

Glucose unit	
DP	**Glc(β1 → 4)**
1	1.000
2	1.810
3	2.488
4	3.523
5	4.832
6	6.342

Fig. 2 compares the separation of a Glc(α1 → 4)$_n$ and a Glc(β1 → 4)$_n$ ladder, which both comprise glucose elements but with α and β linkages, respectively. This linkage difference caused significant migration-time shifts due to their shape differences. As the inset shows, the Glc(α1 → 4)$_n$ ladder is helical, while the Glc(β1 → 4)$_n$ ladder is linear, rendering different hydrodynamic volumes for sugar chains with the same degree of polymerization (DP). Numerical representation of the corresponding GU values for the Glc(β1 → 4)$_n$ oligomers is shown in Table 1.

4. Sample-preparation issues

The main sample-preparation steps for CE analysis of N-linked glycans are shown in Fig. 3. The process starts with the release of sugar moieties from the biopharmaceutical products (both innovative and biosimilars) (Fig. 3A), followed by partitioning the released sugars [e.g., by ethanol precipitation of the remaining polypeptide chain (Fig. 3B)] and fluorophore labeling of the partitioned sugars (Fig. 3C). Glycan release usually utilizes an endoglycosydase peptide N4-(N-acetyl-β-glucosaminyl)asparagine amidase (PNGase-F).

Using the correct pH for the PNGase-F release reaction (pH 7.0) is very important. If the pH of the reaction buffer is too high or too low, it can cause epimerization

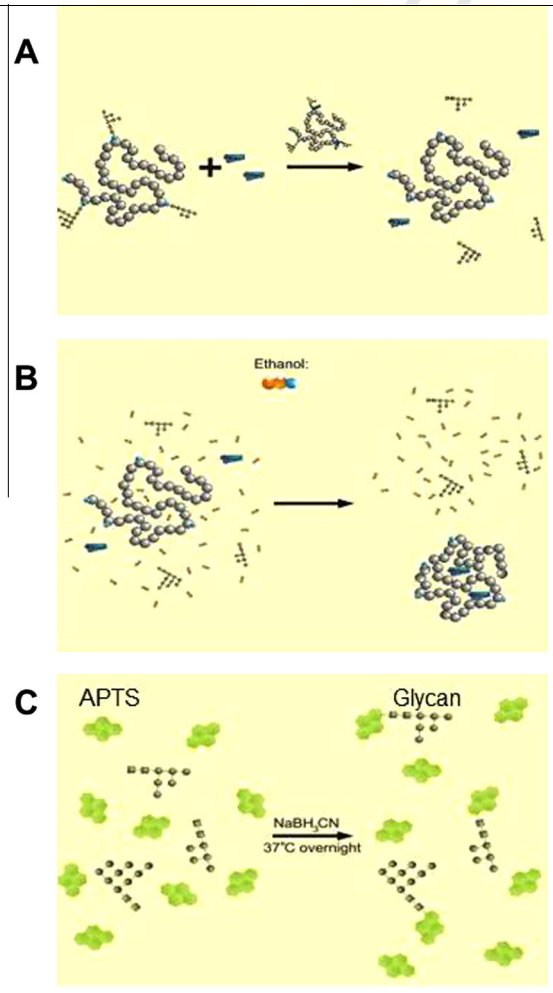

Figure 3. Main sample-preparation steps for capillary electrophoresis analysis of N-linked glycans. (A) Glycan release by PNGase F; (B) sugar partitioning by ethanol precipitation; and, (C) APTS labeling.

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or loss of sialic acids, respectively, which can change the resulting glycosylation pattern [35].

The preferred enzymatic deglycosylation time at 37°C, is 12–16 h. This enzymatic reaction can be accelerated to as fast as 1–2 h at 50°C; however, one should consider the possible loss of labile residues (e.g., sialic acids) at that temperature (50°C), again resulting in a change in glycosylation pattern.

Other options to speed up glycan release are microwave-assisted deglycosylation, immobilized PNGase F enzyme reactors, or pressure-cycling technology, as discussed in [36]. These methods can decrease the release time to as short as just a couple of minutes or even seconds. Please note that other endoglycosidases (e.g., Endoglycosidase H and PNGase A) can also be employed for N-glycan release, if necessary.

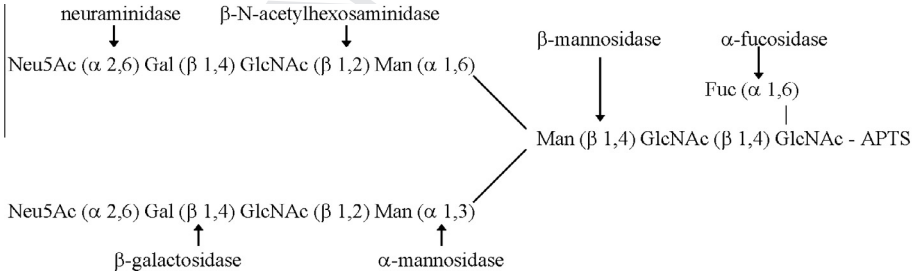
Once the carbohydrate structures are released form the therapeutic glycoprotein, the next sample-preparation step is their labeling by a charged fluorophore. Although a variety of different labeling agents have been suggested in the past [37,38], for the time being, 8-aminopyrene-1,3,6-trisulphonic acid (APTS) [39] is used the most. APTS labeling is a simple reductive amination-based reaction using a weak-acid catalyst (e.g., acetic acid or citric acid), and sodium cyanoborohydrate as reducing agent in organic medium. The lower the pK of the catalyst, the shorter is the reaction time, but, again, strong acidity may raise stability issues for labile sugar residues. The main advantages of reductive amination-based carbohydrate labeling are that the fluorophore only reacts with the reducing ends of sugars in a simple one-step reaction with good derivatization yield (>90%) and negligible structural selectivity [39]. Since only one fluorophore is attached to each glycan structure, the resulting labeled sugars are readily quantified with high sensitivity using ~~detection by LIF or light-emitting diode (LED)~~. Fluorescent labeling is accomplished with a large excess of the labeling reagent, so removal of the unconjugated dye is important, especially when electrokinetic injection is the way to introduce the sample into the separation capillary, as this method causes biased sample entry favoring the labeling reagent. The most common way to remove the excess derivatization dye is by Sephadex G10 resin or normal-phase/HILIC beads [40].

5. Carbohydrate sequencing

Full structural elucidation of glycans, including information about the linkage and the position of the individual sugar residues, is accomplished by carbohydrate sequencing in a step-wise or array manner using specific exoglycosydase enzymes with appropriate sugar and linkage specificity, as shown in Table 2 [41]. In practice, the fluorophore-labeled sugar structures are subject to top-down digestion and bottom-up identification. This means that first one type of sugar residue (e.g., sialic acid, fucose, and GlcNAc) is removed from the non-reducing end of the carbohydrate and the resulting truncated structure is analyzed by CE-LIF. Then, the next sugar-residue types are removed and the resulting profiles analyzed again by CE-LIF, until the N-lined core structure of $GlcNAc_2Man_3$ is obtained. At this stage, all the CE traces are compared and, based on the migration-time shift and time changes of the individual peaks in all the traces, the entire structure can be reconstructed including the position and linkage information of the individual sugar-building blocks. The most frequently used exoglycosidase enzymes are linkage-specific sialid-

Table 2. Exoglycosidase-enzyme array-based carbohydrate sequencing. The lower panel depicts the cleavage spots of the individual enzymes in the matrix. {Published with permission from [41]}

Enzymes/vials	1	2	3	4	5
Neuraminidase (NANase)	x	x	x	x	x
β-Galactosidase (GALase)	-	x	x	x	x
β-N-Acetylhexosaminidase (HEXase)	-	-	x	x	x
α-Mannosidase (MANase)	-	-	-	x	x
α-Fucosidase (FUCase)	-	-	-	-	x



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ases, galacosidases, fucosidases, hexosaminidases and mannosidases [29,41]. It is important to note that sialylated structures have higher charge states due to the number of sialic-acid residues in addition to the three negative charges of the APTS label. These extra charges cause faster electrophoretic migration of these species, resulting in possible comigration of multiple structures at the early migration-time regime of the electropherogram, making structural elucidation extremely challenging and sometimes even impossible.

To alleviate this problem, one can apply preparative weak anion exchange (WAX) chromatography fractionation of the sialylated glycans with the different charge states before the derivatization reaction with the charged fluorophore. This step separates sialo structures (e.g., mono-, di-, tri-, and tetra-) and the fractions are then handled as individual glycan pools (i.e. subject to derivatization, purification and exoglycosidase-array-based glycan sequencing). This method was successfully applied to the analysis of heavily-sialylated biopharmaceuticals (e.g., erythropoietin) [42]. Extra charges on glycans can be caused by other groups (e.g., phosphorylation), in which case immobilized metal affinity chromatography (IMAC) was successfully utilized for their partitioning before the application of the exoglycosidase-based sequencing [43].

6. Glycan analysis during biopharmaceutical development and production

Manufacturing of biotherapeutics includes cloning, protein expression, protein production, purification, product characterization and validation, as shown in Fig. 4. During all of these steps, careful analysis of the protein and its PTMs (e.g., glycosylation) are crucial. The first stage of this process is clone selection for glycoprotein therapeutics, which requires high throughput that usually involves screening hundreds of clones, including analysis of their glycosylation profile. Understanding the sugar-to-function relationship is already critical during the selection of cell lines to assure that it will provide appropriate PTMs for the required function (Quality by Design, QBD). Glycosylation analysis should also be applied in all the following steps of biotherapeutic production; however, ~~the number of samples but the speed of analysis is not then~~ one of the most important factors. Finally, checking for appropriate glycosylation during lot release is the final, crucial glycoanalysis step.

Many factors contribute to alterations in glycan processing on recombinant glycoproteins, including the expression levels of the processing enzymes in the host cell line, monosaccharide nucleotide donor levels, cell-signaling pathways (cytokines/hormones, drugs, media components), loss of cellular organelle organization (e.g., due to pH changes), mutations in genes, gene silencing, overexpression, bioprocessing environment such as temperature, and oxygen level – just to list the important ones. Having the proper analytical toolset is therefore absolutely necessary to ensure that the product possesses correct glycosylation for the expected biomedical activity. Having the proper glycoanalytical toolsets is of contemporary importance, as, in a couple of years, dozens of biotherapeutic drugs will be off patent, so companies will start producing their biosimilar versions [44]. Biosimilars are presumably produced in a same

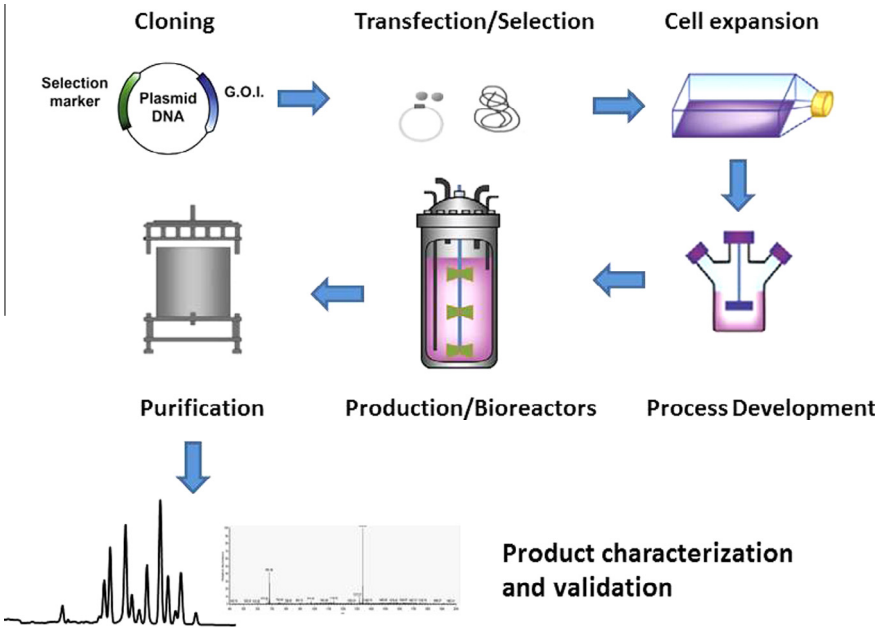

Figure 4. The manufacturing process of recombinant therapeutic proteins where each step requires glycosylation analysis.

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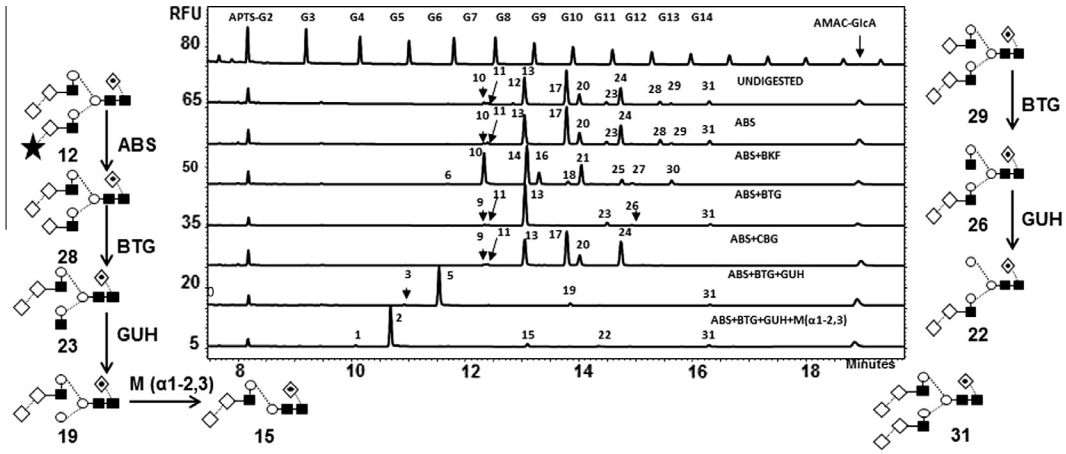

Figure 5. Exoglycosidase digestion based α-1,3-Gal content analysis of an NCI reference standard monoclonal antibody. {Published with permission from [50]}.

way as the innovator products, but usually without exact knowledge of the expression system and production parameters of manufacturing of the innovator product. Thus, the glycosylation pattern of a biosimilar can be very different, changing some of the features in comparison to the innovative compound. Of course, it can also result in a so-called bio-better product, but, by all means, the glycosylation pattern should be carefully analyzed and documented.

The majority of current biopharmaceuticals (and biosimilars) are Ab therapeutics, in particular mAbs of the IgG1 sub-type. IgG1 possesses approximately 2–3% carbohydrate by mass, primarily attached to the highly-conserved N-glycosylation site at ASP 297 in the CH2 domain of the Fc region of each heavy chain [45]. Glycosylation variability on Ab therapeutics depends on the cell line and expression conditions used, possibly leading to structural diversity with respect to their fucose, galactose, sialic acid and N-acetylglucosamine content, influencing its biological activity, physicochemical properties and last but not least the ADCC and CDC functions [46]. ADCC activity, if that is the mode of action of the Ab drug, can be enhanced by decreasing the amount of core fucosylated glycans at ASP 297. N-acetylglucosamine and mannose residues at the same site provide ligands for Mannose Binding Protein. The presence of sialic acids somewhat suppresses ADCC and provides anti-inflammatory features, while galactosylation enhances CDC function.

Besides the QBD considerations, other important aspects of glycomic analysis are the determination of the presence or the absence of potential immunogenic epitopes, even at trace levels. α1,3-gal and NGNA moieties are antigenic, so their level should be very carefully checked throughout the entire manufacturing process.

Please note that additional glycosylation sites may be found in the hypervariable regions of the Fab portion of Ab therapeutics and should be analyzed accordingly [47].

7. Detection of potentially-immunogenic epitopes

It has been well documented that non-human oligosaccharide motifs of galactose-α-1,3-galactose (α-1,3-Gal) and N-glycolylneuraminic acid (Neu5Gc) may trigger immunogenic response in humans [48]. As glycosylation is subject to cell-culture type and conditions, alterations in the process can result in different levels of immunogenic sugars [49], and, since these epitopes may adversely affect the safety of biotherapeutic products, minimizing their levels during product development and production is very important.

Galactose-α-1,3-galactose residues usually get attached to the non-reducing end of glycans. The high immunogenicity of α-1,3-gal is evidenced by ~1% of all human Abs being against this epitope. Fig. 5 depicts a systematic exoglycosidase-array-based approach to α-1,3-gal residue analysis of a reference standard mAb from the National Cancer Institute (NCI) [50]. Consecutive exoglycosidase-digestion steps, including alpha and beta galactosidases, revealed the presence of α-1,3-gal epitopes on the 1-6 arm (left flowchart, structure 12), on the 1-3 arm (right flowchart, structure 29) and in both arms (structure 31) of the antennary structures of mAb glycans. A quantitative study showed almost 10% α-1,3-gal-containing glycan structures in this particular reference material.

N-acetylneuraminic acid (Neu5Ac) and its hydroxylated form, N-glycolylneuraminic acid (Neu5Gc), are the two major sialic acids found in mammals and typically terminate the antennary chains of both N-glycans and O-glycans *via* enzymatic addition by sialyl transferases. Neu5Gc is not expressed in humans due to the evolu-

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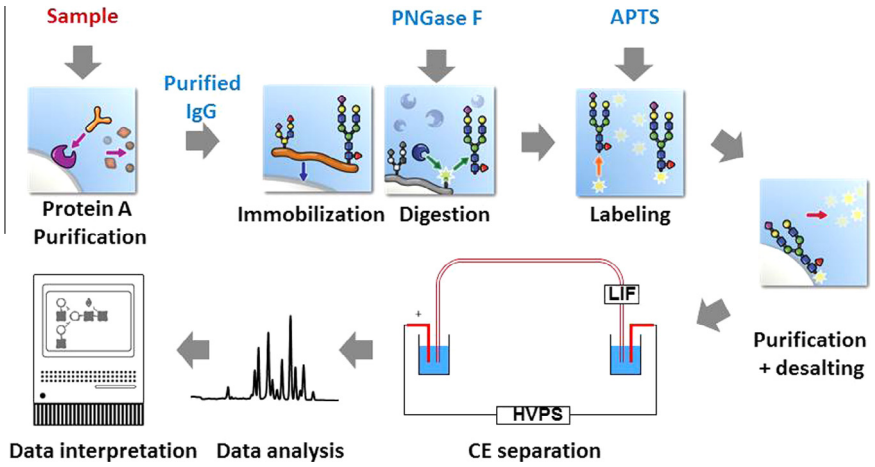

Figure 6. Automated glycan analysis flowchart. {Adapted from [55] with permission}.

tionary loss of the gene encoding the enzyme that converts Neu5Ac into Neu5Gc (CMP-Neu5Ac hydroxylase). ~~Indeed,~~ humans possess circulating Abs against Neu5Gc, so glycans attached to protein therapeutics expressed in cell lines capable of Neu5Gc incorporation can have the associated immunogenic potential [51]. CE analysis of Neu5Gc is usually done at the monosaccharide-analysis level after labeling with a non-charged fluorophore, 2-aminoacridone [52].

8. Conclusions

This article reviewed the increasing role of CE with respect to N-glycosylation analysis of biotherapeutics. Glycosylation analysis is important during all the steps of the biopharmaceutical-manufacturing process. In clone selection, identification of all relevant glycan structures, including relative quantification of the individual carbohydrates, is readily accomplished by CE in a high-throughput manner. CE-LIF also provides the fast turn-around time and high sensitivity that is required during process development to check glycosylation consistency and to detect the presence of possibly immunogenic residues. Full structural elucidation is accomplished by exoglycosydase-array treatment followed by liquid-phase separations or by checking critical glycosylation features with MS. This includes full sequence analysis, purity assessment, quantitation and identification. In formulation development, possible glycosylation changes can be readily monitored by CE. The same applies to comparability studies and release analytics, which both require detection of all sugar structures with high sensitivity and accurate quantification under good manufacturing practice (GMP).

To accommodate the proposal of regulatory agencies to use orthogonal separation methods for the analysis of biotherapeutics, CE is one of the choices, along with other glycoanalytical techniques (e.g., HILIC or MS). The orthogonality of CE-LIF and HILIC-UPLC was recently reported in a comparative study of analyzing fluorophore labeled IgG glycan pools, revealing that the major structural sugar groups eluted/migrated in different positions with respect to their corresponding sugar-ladder standards [53]. For example, while sialylated structures eluted late in HILIC-UPLC, they migrated early in CE-LIF. However, neutral glycans migrated later in CE-LIF, as their charge to hydrodynamic volume ratio was lower, but eluted early in HILIC-UPLC. Approximately the same number of glycans was identified in both techniques.

An automated CE-based glycan-analysis flowchart is shown in Fig. 6. This workflow can be readily applied to the N-glycosylation analysis of the largest group of biopharmaceuticals, mAb therapeutics. The steps include purification of the IgG molecules by protein-A partitioning, followed by PNGase-F digestion, fluorophore labeling, sample purification/desalting and CE separation. The CE-LIF data is then analyzed and interpreted for structural elucidation, and also compared to publicly-available databases [34].

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