High-Throughput Glycoanalysis for Biopharmaceutical Development and Manufacturing

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Glycosylation is the most versatile and one of the most abundant of all co- and posttranslational modifications (**1**). Its structural role affects the solubility of a protein and the stability of its conformation. Glycosylation can affect protein activity and may significantly change a protein's half-life in serum by affecting its clearance by kidneys and protecting it from proteolysis.

Glycosylation is not template-driven and is currently impossible to predict. Glycosylation sites on glycoproteins commonly display microheterogeneity because they are glycosylated by the actions of a series of glycosidases and glycosyltransferases. This results in a number of glycan structures that can vary according to the physiological status of the cells (**2**). Different cell lines and different fermentation conditions can produce significantly different glycosylation patterns (**3–5**).

Recombinant biopharmaceutical proteins are gaining a rapidly increasing share of the pharmaceutical industry (**6**). More than 150 biopharmaceuticals have now gained medical approval, and several hundred are in the pipeline — most of which are glycoproteins produced in mammalian cell systems (**7–10**). Production capacity for recombinant protein drugs often becomes rate limiting to meeting market need, leading to investment of significant effort to develop cell lines capable of producing large quantities of protein. Often, such cell lines and their growth conditions are optimized primarily for protein quantity, which can alter the glycosylation patterns of expressed glycoproteins. Obtaining clones and optimizing growth conditions that will retain desired glycosylation patterns is key to relieving the capacity shortfall for biologics manufacturing. Because glycosylation is highly sensitive to the process of protein-therapeutics manufacturing — the type of host cell, the particular clone chosen, and the growth conditions — there is a growing need for characterization and monitoring of glycan structure at all stages of discovery, development, and manufacturing of protein therapeutics.

Glycoanalysis is a complex and challenging task in producing biopharmaceuticals because of the complexity of the glycans and their biophysical properties. Current glycoanalytical methods rely mainly on chromatographic and mass spectrometry–based methods (Table 1). Those techniques are generally accurate, but they require significant labor and scientific expertise in sample preparation and data analysis. Most methods involve removing the glycans from proteins (**11–13**) and are usually time-consuming, requiring a few days for completion. Moreover, usually more than one technique is required for a complete analysis (**12**). These issues make the use of such technologies impractical for high-throughput monitoring of glycosylation during process development and manufacturing of biopharmaceuticals.

A Lectin-Based Array: Here we describe a new lectin-array–based method for rapid analysis of glycosylation profiles of

Glycoanalysis at work: the GlycoScope software screen
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KEYWORDS: GLYCOSYLATION, BIOPHARMACEUTICALS, LECTIN ARRAYS, MONOCLONAL ANTIBODIES

LEVEL: INTERMEDIATE

Table 1: An overview of commonly available methods for glycoanalysis

Method	Output	Sample preparation procedures	Sample amount	Time (including sample preparation)	Throughput*	Required qualification
Chromatography						
HPLC	Glycan profiling; structural information; quantitative	Removal and purification of glycans from protein; end-labeling	10–100 µg	Days	Low	Highly skilled personnel
Electro-chromatography (CE)	Glycan profiling; quantitative	Removal and purification of glycans from protein; end-labeling	100 µg	Days	Low	Highly skilled personnel
CE based Glycan mapping on DNA sequencer	Glycan profiling; structural information; quantitative	Removal and purification of glycans from protein; end-labeling	1–10 µg	Days	High	Highly skilled personnel
High-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) (Dionex systems)	Simple charge-based profiling; qualitative	Removal and purification of glycans from protein; end-labeling	100–1,000 µg	Days	Low	Highly skilled personnel
GlycoScope	Glycan profiling; quantitative	Intact glycoproteins; no labeling; no digestion	0.1-10 µg	Hours	High	Minimal training
Mass Spectroscopy						
Electrospray ionization (ESI)	Glycan molecular weight; qualitative	Removal and purification of glycans from protein; end-labeling	10–100s µg	Days	Low	Highly skilled personnel
Matrix assisted laser-desorption ionization (MALD)I	Glycan molecular weight; mixture profiling; qualitative	Removal and purification of glycans from protein; end-labeling; Co-crystallization of sample and matrix.	10–100s µg	Days	Low	Highly skilled personnel
Nuclear Magnetic Resonance (NMR)						
1D and 2D methods	Sequence and sterical structural information; qualitative	Cleavage of glycan from protein; highly purified glycans	Several mgs	Weeks–months	Single	Highly skilled personnel

*Relative to glycoanalysis methods throughput

biopharmaceuticals. The array contains almost 30 well-characterized plant lectins with overlapping specificities. Lectins are a family of carbohydrate-recognizing proteins classified into a number of specificity groups based on the monosaccharides for which they exhibit the highest affinity (**14, 15**). Our platform is based on binding of intact glycoproteins, through their glycans, by the arrayed lectins. Glycoprotein binding to the array results in a characteristic fingerprint that is highly sensitive to changes in a protein's glycan composition. The large number of lectins, each with its characterized recognition pattern, ensures high sensitivity to changes in the glycosylation pattern. Automatic algorithms were constructed for interpreting these signals into a quantitative glycan profile output, based on lectin–glycan recognition rules. Figure 1 presents a schematic representation of the process.

Analysis can be performed directly on nonpurified bioreactor supernatant samples, requiring only nM–µM concentrations in low (100–150 µL) volumes. Dozens of samples can be automatically analyzed in parallel in under six hours using an automatic slide processor. The platform enables glycosylation monitoring at all stages of biopharmaceutical development including clone selection, process development, and manufacturing.

Here we present results obtained by comparing our platform with traditional HPLC methods for glycoanalysis of several therapeutic recombinant monoclonal antibodies (MAbs). The results demonstrate our method's applicability to glycoanalysis of biopharmaceuticals during various stages of development and manufacturing.

Glycosylation of Recombinant MAbs

The MAb market is one of the fastest growing and most lucrative sectors of the pharmaceutical industry, with a potential to reach $30 billion in 2010. It is driven by technological evolution from chimeric and humanized to fully human antibodies (immunoglobulin G

Abbreviations Used in This Article

MAb: Monoclonal antibody
Fc: Constant region
G0: 0 Galactose
G1: 1 Galactose
G2: 2 Galactose
Gal: Galactose
GalNAc: N-acetyl galactosamine
Glc: Glucose
GlcNAc: N-acetyl glucosamine
GS: GlycoScope
HPLC: High- performance liquid chromatography
IgG: Immunoglobulin G
Man: Mannose
MS: Mass spectrometry
N: Aspargine
Term GlcNAc : Terminal N-acetyl glucosamine

Figure 1: GlycoScope glycoanalysis platform process flow

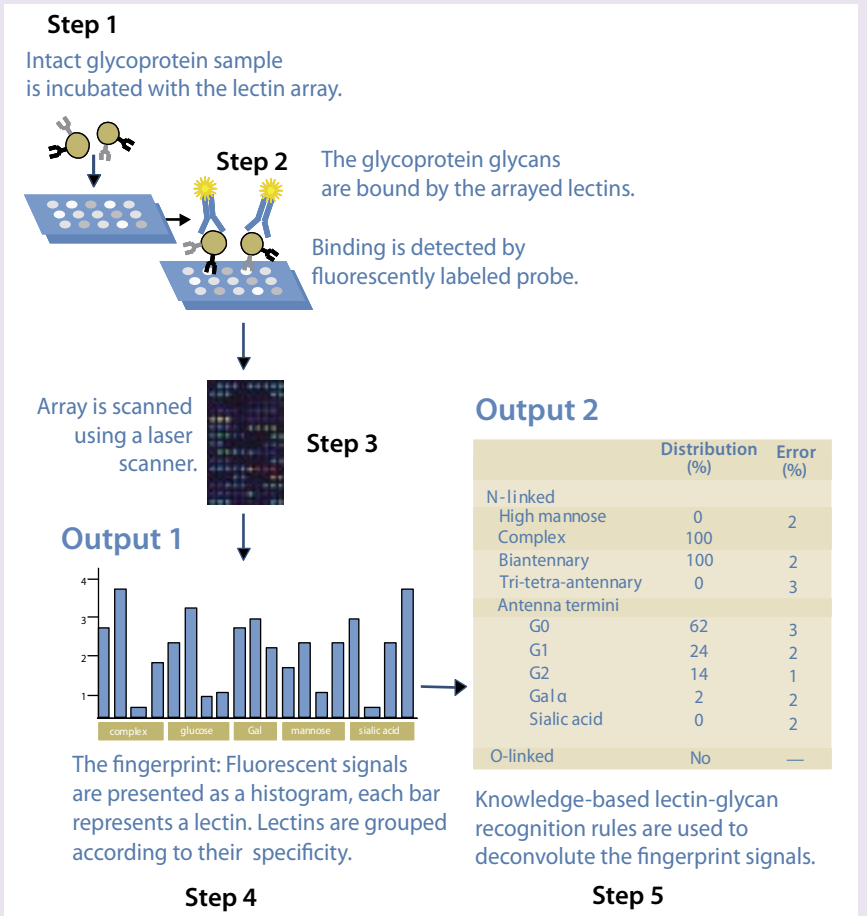


Table 2: Quantitative glycoanalysis of monoclonal antibody samples displaying different glycan types

	MAb1		MAb2		MAb3		MAb4	
Glycan structure (%)	**GS**	**HPLC**	**GS**	**HPLC**	**GS**	**HPLC**	**GS**	**HPLC**
High mannose	ND	0	ND	0	ND	0	21 ± 0	17
Complex								
Biantennary	100 ± 0	100	100 ± 0	100	100 ± 0	100	79 ± 0	83
Tri-tetra-antennary	0 ± 0	0	0 ± 0	0	0 ± 0	0	0 ± 0	0
Antenna termini								
G0*	28 ± 6	25	51 ± 3	54	68 ± 5	67	60 ± 11	48
G1*	50 ± 1	50	41 ± 2	39	29 ± 4	28	35 ± 8	40
G2*	22 ± 5	25	8 ± 1	6	3 ± 1	5	5 ± 3	12
Gal (α 1–3) Gal	0 ± 1	0	0 ± 0	0	2 ± 1	0	1 ± 1	0
Sialic acid	ND	0	ND	0	ND	0	ND	0

*Calculated as percent of biantennary glycans; ND = not detected

type, primarily, IgG). The number of therapeutic MAbs under development is growing annually by 25% and is expected to exceed 800 molecules by 2010 (**16**).

IgG is a glycoprotein containing at least two N-linked glycosylation sites. The Fc region is glycosylated with predominantly three types of N-linked complex biantennary glycans containing zero, one, or two galactose residues on their outer arms, commonly known as G0, G1, and G2, respectively (**17**). Glycosylation of the IgG–Fc is essential for optimal expression of biological activities, because the glycan composition influences its effector functions mediated through Fc receptors and the C1q component of the complement system (**18**). Glycosylation is therefore essential in production of therapeutic antibodies, limiting manufacturing to mammalian systems. Commonly used systems include rodent cell lines and recently also transgenic animals. Major efforts are invested in developing recombinant antibodies that display desired glycosylation structures, including genetic manipulation of glycosylation enzymes in cells producing the antibodies and optimization of media and growth conditions.

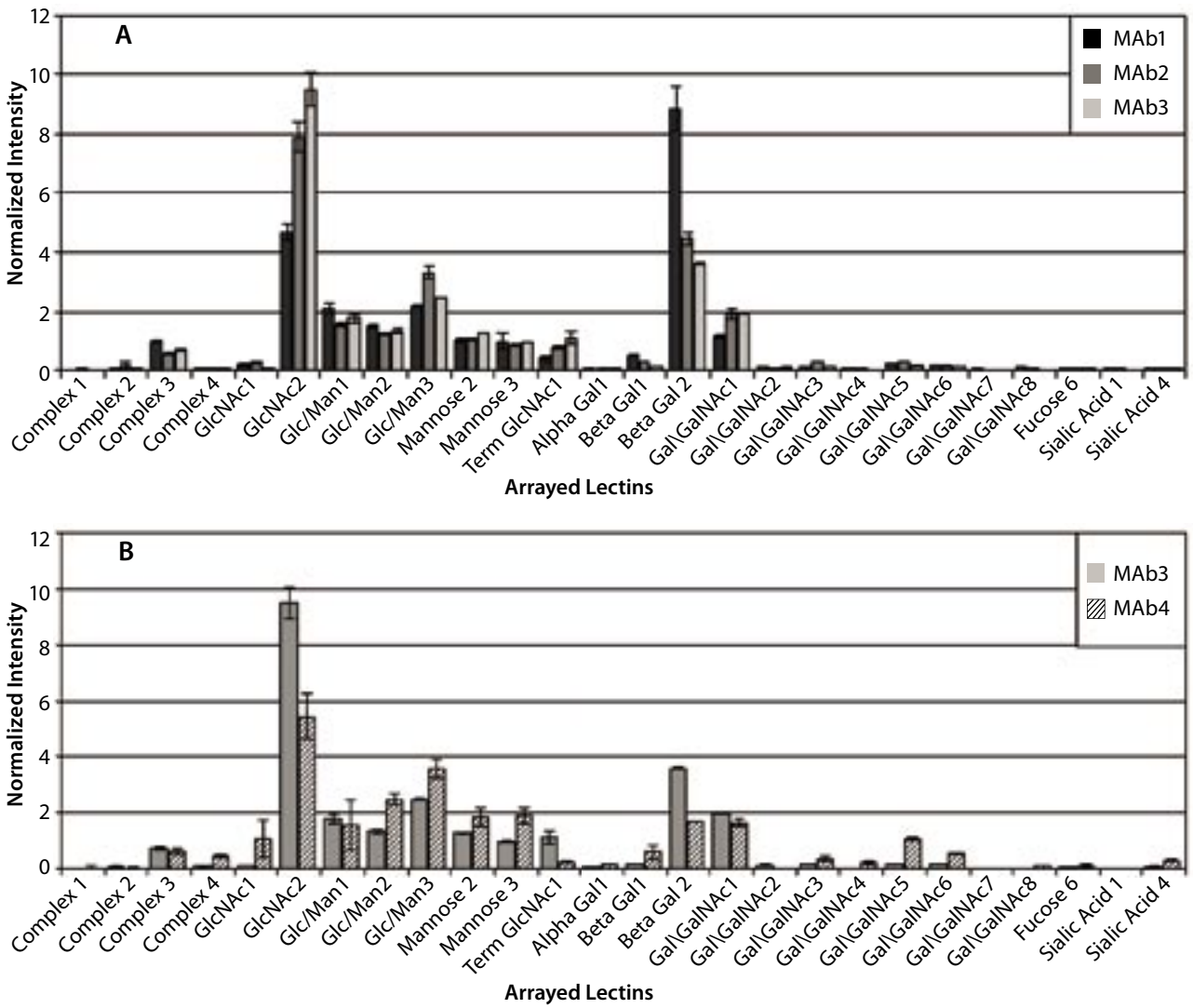
We applied our lectin-array–based platform (GlycoScope) to glycoanalysis of recombinant MAbs produced in mammalian cell lines. The method provides quantitative data for various structures of Fc glycans, including high mannose, complex glycans antennarity, antenna truncation level (G0, G1, G2), the antigenic epitope Gal (α1–3) Gal, and sialic acid. The limit of detection is 5% for most epitopes, except for sialic acid and high mannose (for which the limit of detection is 10%).

Because of the tertiary structure of IgG, the Fc glycans are not completely accessible to all lectins. To allow access of lectins on the array to the Fc glycans of IgG, a short and simple exposure protocol for mild denaturing of the glycoprotein is applied on the lectin array before analysis. This protocol is calibrated for each type of antibody, and it can take 15 minutes to two hours.

Glycoanalysis of Recombinant MAbs with Varying Glycan Types: To demonstrate the applicability of our platform in analyzing MAbs with different glycosylation patterns, we chose four recombinant MAbs manufactured by different manufacturers. These four antibodies represent a broad range of glycosylation patterns commonly found on therapeutic MAbs manufactured in mammalian cells.

Table 2 presents results comparing our method with HPLC analyses. The results demonstrate the accuracy of data obtained for the glycan structures of these MAbs: For most epitopes, those data are within a few percents of the data obtained by HPLC analysis. The only exception is the accuracy of the G0, G1, and G2 predictions in the presence of high mannose structures

Figure 2: Fingerprints of monoclonal antibodies displaying different glycan types[a]



[a] Fingerprints (A) of MAb1–3; (B) fingerprints of MAb3–4. Purified MAb (IgG1) products were diluted with sample buffer to a final concentration of 30 µg/mL (0.2 µM). 200 µL of the diluted sample was subjected to treatment for Fc-glycan exposure before incubation with the lectin array. Binding of the MAb protein to the lectin array was detected using a fluorescently labeled anti-IgG polyclonal antibody. The array was scanned and fingerprints were produced using proprietary algorithms. The fingerprint data was used to calculate the quantitative list of glycan structures presented in Table 2. Results are calculated as the relative abundance of each structure within each group of structures.

Each group is calculated independently. Group 1: N-linked complex antennarity; Group 2: G0-, G1-, G2-type biantennary glycans; Group 3: Gal (α1-3)Gal epitope, calculated independently of G0/G1/G2 ratio; sialic acid is provided qualitatively as detected/not detected. HPLC analysis was performed as follows: IgGs were denatured and trypsinized. N-glycans were released from glycopeptides by PNGase F digestion, purified on Sep Pak and Whatman, and labeled at the reducing end with a fluorescent probe (2AB). The oligosaccharide profile is recorded by normal phase chromatography with fluorescence detection.

(MAb4). In this case, the maximum difference between the methods is 12%. The presence of a percentage of high-mannose structures appears to affect the accuracy in quantification of the G0, G1, and G2 predictions (MAb4); however, the overall G0/G1/G2 ratio is maintained.

The quantitative list of the glycan epitopes presented in Table 2 is calculated by our platform's software from the fingerprint, which is the graphic representation of the binding pattern of the sample to the array (Figure 2). The fingerprints of the four MAb samples demonstrate several differences in sample binding to the lectin arrays. For example, decreasing galactose levels in MAb1, MAb2, and MAb3 samples, respectively, are demonstrated by the decreasing signals of the beta galactose lectins (Beta Gal1 and Beta Gal2 lectins) and a consistent increase in intensities observed for the lectins that preferentially bind glycans with low galactose content (GlcNAc2 and Term GlcNAc1 lectins, as shown in Figure 2A).

The presence of high-mannose–type glycans in MAb4 is demonstrated by comparing its fingerprint with that obtained for MAb3, which does not contain high-mannose–type glycans (Figure 2B). The fingerprints demonstrate stronger binding by lectins that preferentially bind mannose residues (Glc/Man 1–3, Mannose 2–3) and consistently lower signals of the lectins that preferentially bind GlcNAc or galactose-containing antennae (GlcNA2, Term GlcNAc1, Beta Gal2).

Glycosylation of Recombinant MAbs Produced by Different Cell Lines: A different recombinant human IgG1-

Table 3: Quantitative glycoanalysis of MAb5 samples produced by different cell lines

Glycan structure (%)	MAb5 from NS0		MAb5 from Sp2/0	
	GS	HPLC	GS	HPLC
High mannose	NA	3	NA	2
Complex				
Biantennary	100 (RSD = 0)	100 (RSD = 0)	100 (RSD = 0)	100 (RSD = 0)
Tri-tetra-antennary	0 (RSD = 0)	0 (RSD = 0)	0 (RSD = 0)	0 (RSD = 0)
Antenna termini				
G0*	28 (RSD = 10)	33 (RSD = 3)	66 (RSD = 2)	74 (RSD = 4)
G1*	50 (RSD = 1)	45 (RSD = 1)	31 (RSD = 2)	24 (RSD = 12)
G2*	22 (RSD = 11)	21 (RSD = 6)	4 (RSD = 1)	2 (RSD = 26)
Gal (α 1–3) Gal	4 (RSD = 32)	3 (RSD = 6)	1 (RSD = 1)	1 (RSD = 63)
Sialic acid	ND	ND	ND	ND

*Calculated as percent of biantennary glycans; ND = not detected; NA = not analyzed; RSD = relative standard deviation.

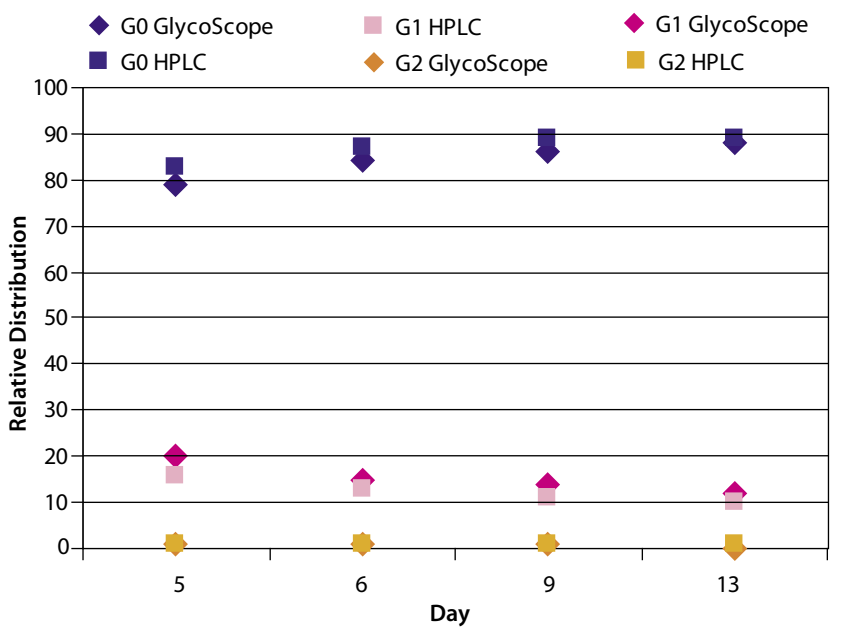
Note: The provided results are averaged from six independent runs of NS0 samples and four independent runs of SP2/0 samples.

Table 4: Glycoanalysis of MAb6 samples harvested on different fermentation days

Glycan structure (%)	GlycoScope				HPLC			
	Day 5	Day 6	Day 9	Day 13	Day 5	Day 6	Day 9	Day 13
High mannose	NA	NA	NA	NA	ND	ND	ND	ND
Complex								
Biantennary	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100	100	100	100
Tri-tetra-antennary	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0	0	0	0
Antenna termini								
G0*	79 ± 4	84 ± 4	86 ± 4	88 ± 5	83	87	89	89
G1*	20 ± 4	15 ± 4	14 ± 4	12 ± 4	16	13	11	10
G2*	1 ± 1	1 ± 0	1 ± 0	0 ± 0	1	1	1	1
Gal (α 1–3) Gal	0 ± 2	0 ± 2	3 ± 3	0 ± 0	0	0	0	0
Sialic acid	ND	ND	ND	ND	ND	ND	ND	ND

*Calculated as percent of biantennary glycans; ND = not detected; NA = not analyzed

Figure 3: Changes in Fc glycosylation during fermentation: graphic representation of the G0/G1/G2 ratios obtained by GlycoScope and HPLC methods for MAb6 glycans[a]



[a] MAb6 (IgG1 produced in NS0) supernatant samples were harvested on different days during fermentation and purified with Protein A columns conditioned with Na-phosphate pH 7.0. The samples are applied onto the columns, washed with Na-phosphate, and eluted with acetic acid. Purified samples were analyzed by GlycoScope and HPLC as described for Figure 2. The detailed analysis from which the data is taken is presented in Table 4.

type MAb, developed for therapeutic applications, MAb5 was grown in NS0 and SP2/0 hybridoma cells. Both cell lines are commonly used for manufacturing recombinant MAbs. Table 3 presents our glycoanalysis results as the relative abundance of each epitope (%) and compared with the data obtained by HPLC to demonstrate our method's accuracy.

Glycoanalysis results obtained by the two methods are highly similar. Both methods revealed major differences in the glycosylation patterns of the Fc glycans of MAb5 produced by the two cell lines. Significantly higher galactose levels are detected for the antibody grown in NS0 cells, resulting in a relatively high abundance of G1 and G2 structure. However, the antibody produced in SP2/0 cells contains mainly truncated structures with an exceptionally high percentage of G0 glycans. Small differences are observed in the Gal (α1–3) Gal antigenic epitope levels produced by the NS0 and SP2/0 cells.

Changes in Glycosylation During Fermentation: As fermentation progresses, changes often occur in such conditions as cell density, cell debris, oxygen levels, concentrations of nutrients, and pH. To monitor the effect of changes in fermentation conditions on the glycosylation pattern of Fc glycans MAbs, we analyzed four samples of another antibody, MAb6 (IgG1 from NS0), harvested on different days of fermentation (days 5, 6, 9, and 13 of fermentation). The antibodies were purified with protein A before analysis and analyzed in parallel by our platform and by HPLC. Data obtained by both methods are highly similar, showing a trend of decrease in galactose levels as fermentation progresses (Table 4, Figure 3).

Discussion

Glycosylation is highly sensitive to the processes of protein-therapeutics manufacturing: The type of host cell, the particular clone chosen, and the growth conditions all affect the glycosylation of the products. The industry is facing a growing need for characterization and monitoring of

glycan structure at all stages of discovery, development, and manufacturing of protein therapeutics. The FDA, through its PAT initiative (www.fda.gov/cder/OPS/PAT.htm), is encouraging innovator companies to monitor all parameters that "guide" the quality of proteins. Because glycosylation is an important parameter for a final product's stability, activity, and safety, monitoring it during development of a biopharmaceutical product is critical for the implementation of the PAT initiative.

Monitoring and characterizing glycosylation at early stages of biopharmaceutical development is impractical using conventional analytical methods because limited amounts of sample are available and purification and labor- and time-intensive sample preparation steps are required. Glycoanalysis during clone selection and process development stages therefore presents the industry with an opportunity for improving throughput and efficiency of glycoanalysis methods.

Our platform can monitor glycosylation during an entire life cycle of a glycosylated therapeutic protein. Because only small amounts of unpurified glycoprotein are required, glycosylation monitoring can begin at much earlier stages of clone screening and selection than is possible with currently used technologies. In downstream process development, growth conditions can be chosen by monitoring the effects of media choices and fermentation conditions on glycosylation. During this stage, monitoring enables process engineers to develop a matrix correlating process and glycosylation changes. In upstream process development, certain purification methods may create bias toward particular glycoforms.

Analysis of glycan profiles throughout purification steps enables an educated selection of purification methods. Such a matrix can be used to make predictions about the effects of future process changes on glycosylation. Selection of purification protocols can also benefit from monitoring of fractions after each step. Finally, in-process monitoring of fermentation during manufacture can allow early disposal of batches that are unlikely to meet release specifications because of aberrant glycosylation.

Even greater value can be expected by use of the method during development and manufacturing of generic biopharmaceutical products (biogenerics or biosimilars). Guided selection of cell lines, clones, growth conditions, and purification methods to produce glycosylation patterns similar to a reference product would increase chances for biosimilarity. This should lead to significant savings in product development and clinical trials costs, manifested also in an imperative decrease in time-to-market.

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