DSA-FACE

High-Throughput Analysis of the N-Glycans of NS0 Cell–Secreted Antibodies

L. Defrancq, N. Callewaert, J. Zhu, W. Laroy, and R. Contreras

Glycosylation of therapeutic proteins produced by various eukaryotic host systems is important not only for their biological function (**1**, **2**), but also for pharmacokinetics (**3**) and antigenicity (**4**, **5**). One widely used host system for therapeutic antibody (Ab) production is a murine myeloma cell line referred to as NS0. Some commercial monoclonal antibodies (MAbs) have been produced from NS0 host cells — including Synagis brand (palivizumab) from MedImmune, Inc. (www.medimmune.com) — and many others are in various development stages. Unlike Chinese hamster ovary (CHO) cells (**6**) and human cells, NS0 is known to add the nonhuman antigenic sugar residue Galα1-3Gal (**5**) onto the N-glycans of proteins it makes (**7**). Although no evidence yet proves that the presence of Galα1-3Gal epitopes on recombinant IgG is immunogenic to humans, regulatory agencies might express concerns about therapeutic glycoproteins containing Galα1-3Gal (**8**).

One way to reduce Galα1-3Gal content on NS0 glycoproteins is by some kind of metabolic control during fermentation process optimization using, for example, different media components (**9**, **10**). Alternatively, screening for NS0 clones that exhibit a low incidence of Galα1-3Gal because of their genetic make-up is another way to reduce antigenic content. That would allow selection of more stable and scalable clones and could alleviate the optimization step in downstream fermentation. Both approaches can be tedious and time consuming, making desirable a high-throughput method to quantify Galα1-3Gal content.

Analytical tools for determining glycan structure have become more advanced in recent years (**11–14**). To screen a large number of NS0 process samples for low Galα1-3Gal content in Ab molecules they have produced, a desirable analytical method would offer

- sufficient throughput
- high sensitivity so that the screening can be performed on small-scale cell cultures (preferably 96- or 24-well tissue culture plates)
- the ability to resolve and quantify glycan linkage isomers without additional enzymatic treatments.

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Current methods using mass spectrometry (**14**) are sensitive and can be put to high-throughput use but are generally unable to differentiate linkage isomers without additional enzymatic treatments. In Ab glycosylation, that poses a problem because many Ab molecules are ß-1,4-galactosylated on only one arm of the biantennary core structure. Substitution of a single ß-1,4-linked galactose with an α-1,3-linked galactose would give the same mass as a desirable glycan in which both arms are ß-1,4-galactosylated. That can lead to a underestimation of the extent of α-1,3-galactoylation of the Ab glycans.

PRODUCT FOCUS: ANTIBODIES MADE BY NS0 CELL CULTURE

PROCESS FOCUS: CELL LINE AND PROCESS OPTIMIZATION

WHO SHOULD READ: R&D AND MANUFACTURING PERSONNEL

KEYWORDS: IMMUNOGENICITY, ANTIGENS, NS0 CELL CULTURE, DNA ANALYSIS, BIOANALYTICAL METHODS

LEVEL: ADVANCED

In our study, therefore, we explored a glycan analytical method that uses standard DNA-sequencing equipment (11) to examine the glycosylation of a recombinant MAb derived from different NS0 clones. We were able to resolve the relevant isomers and quantify the Galα1-3Gal content on 500 ng of the MAb.

Materials and Methods

Similar cultured media samples (1 mL) containing a MAb plus 1% fetal bovine serum (FBS from Hyclone, www.hyclone.com) were collected from different NS0 clones and stored at –20 °C until the assay could be performed.

Purification: We used protein A resins from Sigma-Aldrich (www.sigmaaldrich.com) to purify rapidly the impurities in our NS0 culture samples with Eppendorf tubes. You can also use protein A minicolumns (also from Sigma-Aldrich) to purify crude samples. Our major concern was bovine IgG present in the culture media (+1–10% FBS) that could copurify along with the Ab of interest. That problem was resolved by differential elution at acidic pH: After binding to protein A, we first washed the protein resins using pH 5 sodium acetate buffer (100 mM) to remove bovine IgG, then eluted the therapeutic Ab using a pH 3 elution buffer (50 mM sodium citrate).

N-Glycan Profiling: Eluates of protein A affinity columns containing the MAbs were incubated with RCM buffer (8 M urea, 360 mM Tris, pH 8.6, 3.2 mM EDTA) from EMD Biosciences (www.emdbiosciences.com) at 50 °C for an hour to denature the protein. Subsequently, those mixtures were loaded into the wells of a Multiscreen-IP plate from Millipore (www.millipore.com) prepared as described previously (15). Reduction, iodoalkylation, and deglycosylation steps were performed according to reported procedures (15). N-glycan derivatization with 8-amino-1,3,6-pyrenetrisulfonic acid (APTS) and removal of excess free label were achieved as described recently (11).

Briefly, the deglycosylation mixture was evaporated to dryness, and a 1-mL 1:1 mixture of 20 mM APTS from Molecular Probes (www.probes.com) in 1.2 M citric acid and 1 M $NaCNBH_3$ in DMSO was added. Derivatization took 18 hours at 37 °C. After that, the reaction was quenched by the addition of 10 mL of deionized water. Excess (unreacted) APTS was removed using a bed of Sephadex G10 packed in a Multiscreen filterplate from Millipore. After sample application, the resin beds were eluted three times by the addition of 10 mL of water and a 10-second centrifugation at 750*g* in a table-top centrifuge from Eppendorf (www.eppendorf.com) equipped for handling 96-well plates. The eluate was evaporated to dryness, and the derivatized glycans were reconstituted in 5 mL of deionized water.

For exoglycosidase digestion reactions, 1-mL batches of the cleaned-up, derivatized N-glycans were transferred to 250-mL tubes for polymerase chain reaction (PCR). The following digestions were performed by overnight incubation at 37 °C in 10-mL 20-mM sodium acetate at pH 5.0: *Arthrobacter ureafaciens* sialidase (2 units/mL from Boehringer Ingelheim, www.boehringer-ingelheim.com), *Diplococcus pneumoniae* ß-1,4-galactosidase (1 unit/mL, Boehringer Ingelheim), and bovine epididymis α-fucosidase (0.5 units/mL from Glyko, www.glyko.com). Unit definitions are as specified by the enzyme suppliers. After digestion, each sample was evaporated to dryness and then reconstituted in 1 mL of deionized water. Green

Table 1: Percentage of Galα1-3Gal epitope over total monoclonal antibody (MAb) N-glycan from different NS0 clones.

Crude Sample	α-1,3-galactosylated Glycans
A1	7.10 %
A2	6.24 %
A3	14.60 %
A4	9.65 %
A5	12.85 %
B1	7.66 %

Figure 1: Identification of the different glycan structures present. To identify the different glycans, several digestions were performed. Panel A: dextran. Panel B: nondigested sample. Panel C: *Arthrobacter ureafaciens* sialidase. Panel D: *Arthrobacter ureafaciens* sialidase + *Diplococcus pneumoniae* ß-1,4-galactosidase. Panel E: Bovine kidney α-fucosidase. The corresponding structures are illustrated in Figure 2. Similar structures, not bearing a fucose, correspond with underlined numbers to indicate the fucose is missing (e.g., 3/3′).

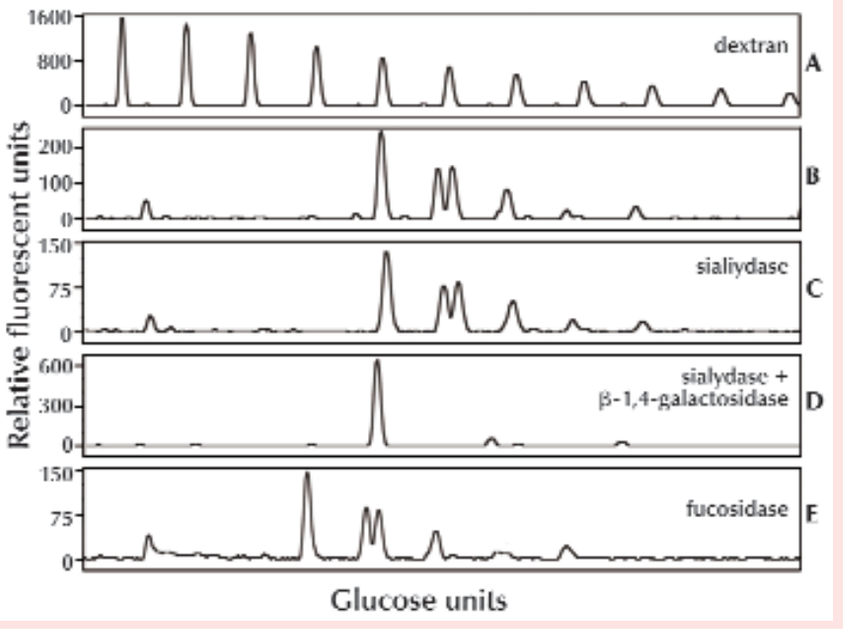
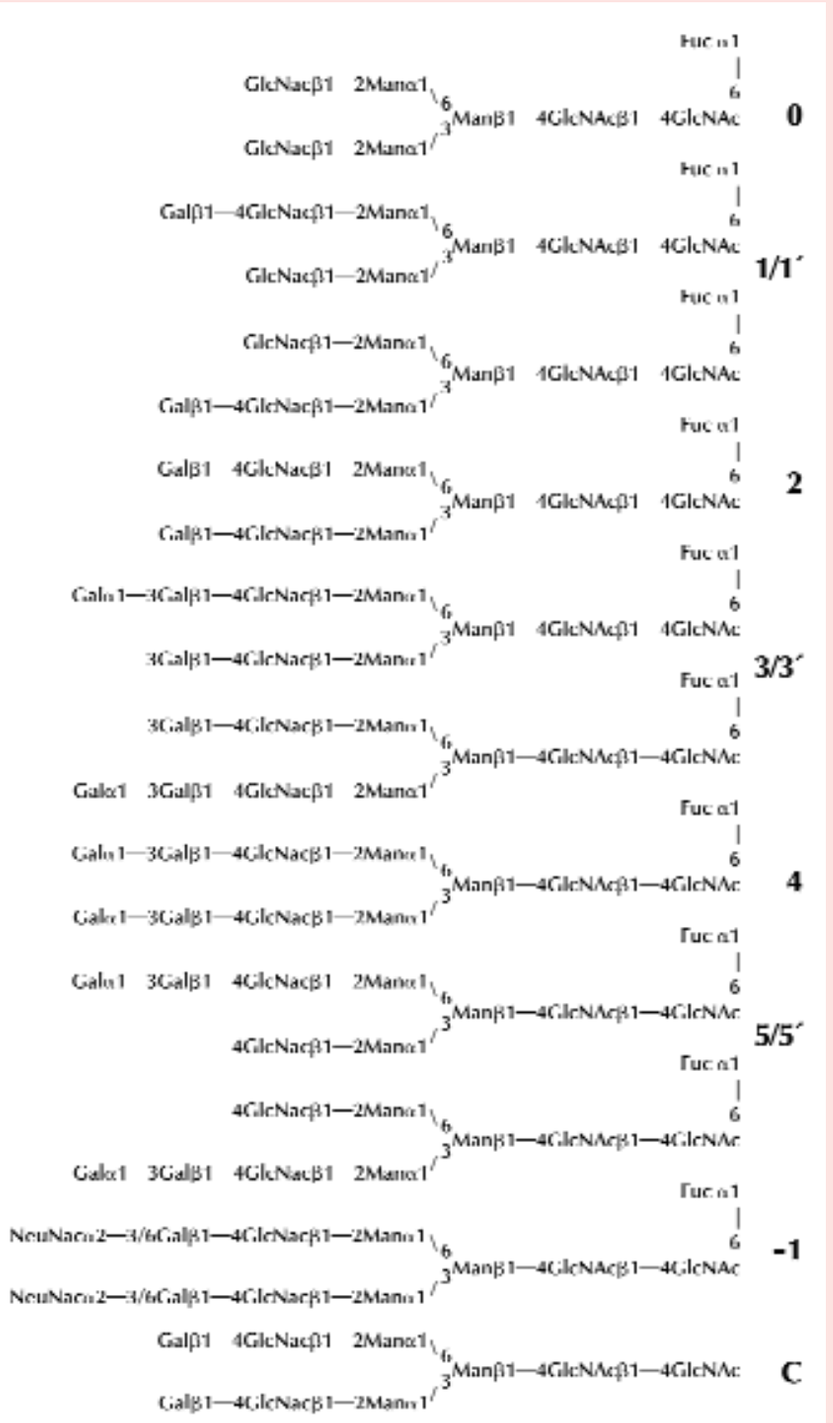


Figure 2: Identification of the peaks in the profile. The structures corresponding with –1, 0, 1/1′, 2, 3/3′, 4 and 5/5′ are present in the sample (Figure 1). C is a reference sugar that was used to serve as a positive control for green coffee bean α-1,3-galactosidase.



coffee bean α-1,3-galactosidase (Sigma) was used under standard conditions: overnight incubation at 37 °C in 10 µL of 20-mM sodium acetate at pH 5.5.

The assay for α-1,3-galactosyltransferase was performed as follows: A biantennary bigalactosylated structure from Glyco, NA_2, was used as acceptor for galactosyl transfer by Calbiochem α-1,3-galactosyltransferase from EMD Biosciences and UDP-Gal (50 mM) from Boehringer Ingelheim. The reaction was performed overnight at 25 °C in a 100-mM sodium cacodylate buffer with 20 mM $MgCl_2$ at pH 6.5. It resulted in a mixture of mono- and bi-ß-galactosylated structures (Figure 3). Before gel-loading the next day, we added 0.5 mL of the rhodamine-labeled Genescan 500 standard mixture based on an internal standard from Applied Biosystems (www.appliedbiosystems.com) and 1 mL of deionized formamide from the same source to each sample.

All experiments were performed on an Applied Biosystems 377A DNA-sequencer adapted for cooling as described (11). The 36-cm gel contained 10% of a 19:1 mixture of acrylamide and bisacrylamide (89 mM Tris, 89 mM borate, 2.2 mM EDTA) from EMD Chemicals Inc. (www.emdchemicals.com). Prerunning was at 3000 V for one hour. The electrophoresis voltage during separation was 3500 V, and data were collected for three hours (with separation of glycans up to 15 glucose units in size). Data analysis was performed using Genescan version 3.1 software from Applied Biosystems. Using the peak positions of the internal rhodamine–oligonucleotide standard, all lanes on the same gel were aligned with that containing the APTS-labeled malto–oligosaccharide standard. After alignment, samples on different gels can be easily and reliably compared by aligning their malto–oligosaccharide positions. For clarity — and to allow black-and white reproduction of the figures presented in this paper — peaks corresponding to the rhodamine-labeled internal standards were omitted after alignment.

Results

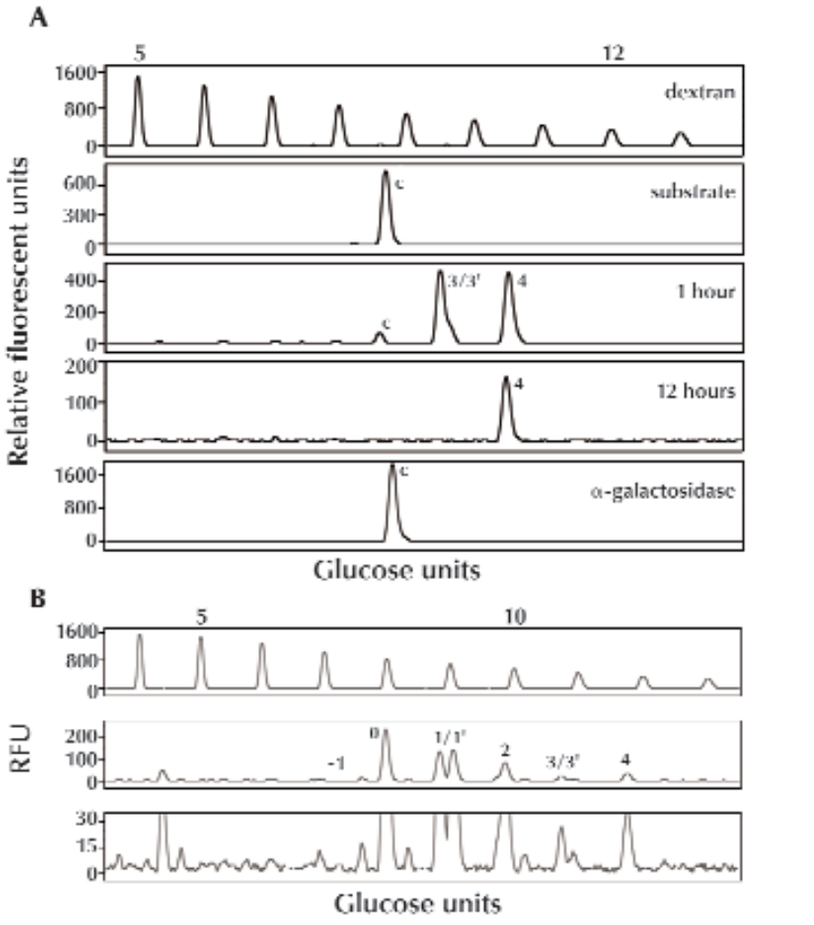
Identification of Peaks in the Profile: Figure 1 shows the size profile of a typical antibody N-glycan sample from NS0 cells. To identify different glycan structures, we performed several exoglycosidase digestions. Sialidase treatment shows that few structures are sialylated because only a minor peak disappears (peak –1, panel C). Upon further ß-1,4-galactosidase digestion, most peaks

shifted to the α-galactosylated biantennary structure (peak 0). So the most common structures are 0, 1/1′, and 2. The behavior of the three remaining peaks (5/5′ and 4) upon ß-1,4-galactosidase digestion is compatible with their being α-1,3-galactosylated structures (see below) because α-1,3-galactosyl substitution of a ß-1,4-galactose residue blocks its removal with ß-1,4-galactosidase. Thus, glycan 4 (with both branches α-1,3-galactosylated) is completely resistant to ß-1,4-galactosidase digestion, and glycans 3/3′ shift about one glucose unit upon ß-1,4-galactosidase digestion (to become glycans 5/5′), indicating the loss of one galactose residue (the other one being blocked by α-1,3-Gal substitution).

Upon α-fucosidase digestion, the whole profile shifts one glucose unit to the left, so all structures bear one fucose residue. The size of that shift upon defucosylation (slightly more than one glucose unit) leads to our conclusion that the fucose is linked to the proximal GlcNAc residue at the reducing terminus of the N-glycans, but not to the branch GlcNAc residues (substitutions give a shift in mobility of 0.7–0.8 glucose units). In conclusion, the recombinant antibodies studied here are N-glycosylated with a mixture of core-fucosylated biantennary glycans corresponding with structures –1, 0, 1/1′, 2, 3/3′, 4 (Figure 2).

To confirm the identity of the α-1,3-galactosylated structures, we needed a reliable α-galactosidase. To test the enzyme, we prepared a control glycan structure by transferring galactose using recombinant α-1,3-galactosyltransferase on the highly purified biantennary reference sugar, C (Figures 2 and 3). After one-hour enzymatic transfer, a mixture of mono- and bi-α-1,3-galactosylated structures (comigrating with peaks 3/3′ and 4 in Figure 3A, lane C) was obtained. Overnight reaction converted the structure completely to the bi-α-1,3-galactosylated form (peak 4, Figure 3A, lane D). Digestion with the α-galactosidase isolated from green coffee beans converted the product back to its original structure C (Figure 3A lane E).

Figure 3: Verification of isomer separation by DSA-FACE. **3A:** A biantennary glycan structure C was α-1,3-galactosylated by bovine recombinant galactosyltransferase. This produced a mixture of structures c, 3/3′, and 4 after a one-hour reaction and a full conversion to structure 4 after a 12-hour reaction. α-galactosidase digestion converted it back to its original form. The corresponding structures are shown in Figure 2. **3B:** N-glycan profile of a MAb (full-scale and 10-fold blowup to visualize the low-abundance species). The corresponding structures are shown in Figure 2.

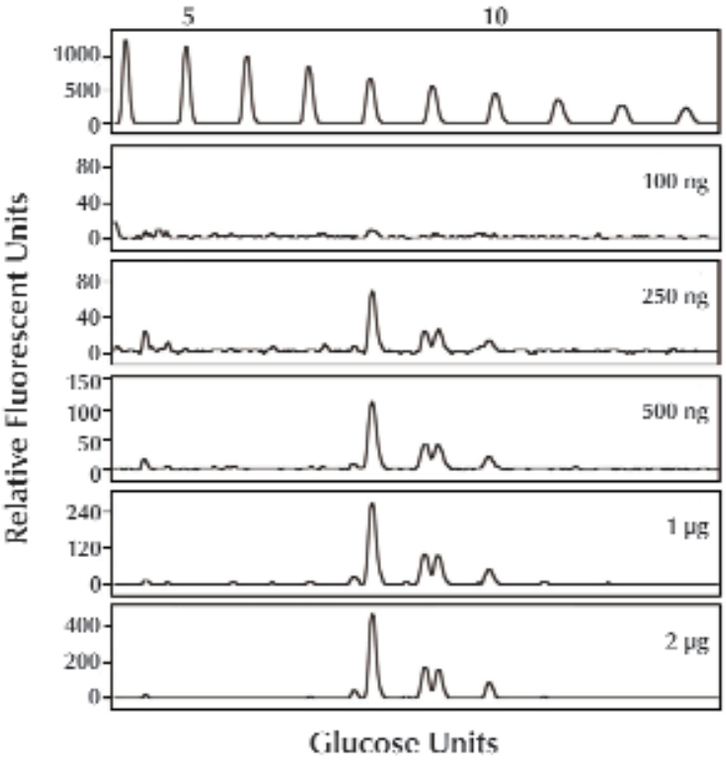


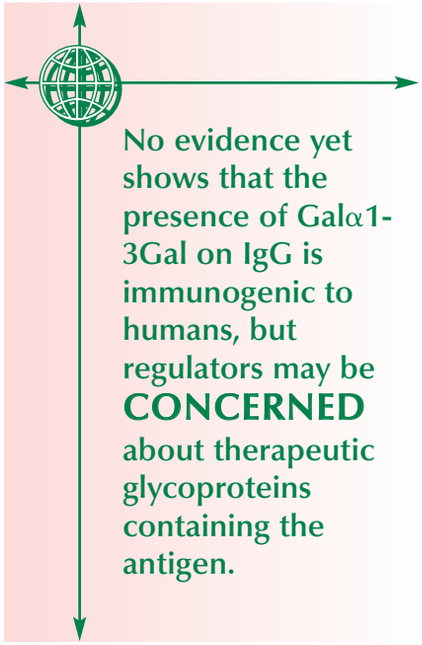
Importantly, the results described in Figure 3A and 3B demonstrate that the resolution of carbohydrate electrophoresis on standard DNA-sequencing equipment is sufficient to discriminate between the two α-1,3 monogalactosylated forms, which is currently all but impossible by mass spectrometry because both isomers have the same mass. To further verify the α-1,3-Gal linkages, α-galactosidase digestion was also performed on antibody samples, which supported the same conclusion (data not shown).

Terminal α-1,3-Galactose Content of N-Glycans on Different Ab Clonal Batches: Starting from minimal amounts of the recombinant Ab (±1 mg), we determined the Galα1-3Gal content even in those cases where there were only minute amounts (~2%) of α-1,3-galactosylated glycans present. If the quantification of low Galα1-3Gal content is not required, the assay sensitivity can come down to as low as 250 ng of the antibodies (Figure 4). From the peak areas, α-1,3-gal content was calculated as in Table 1, varying between 6% and 15% of the glycans.

Because ß-1,4-galactosylated branches are the substrates of α-1,3-galactosyltransferase, we were interested in determining the relation between the extent of ß-1,4-galactosylation and the level of α-1,3-galactosylation. The results

Figure 4: Sensitivity of the assay: To determine the minimum amount of antibody necessary to obtain an N-glycan profile, we analysed a dilution series (0.1–2.0 µg) of a purified MAb. The results show that 250 ng of antibody protein is sufficient to generate a profile that allows quantification of the main N-glycans. However, 2 µg of the protein is needed to detect the N-glycans representing 1% of the total pool with a signal-to-noise ratio of >3.





of our analysis clearly show a linear relationship between the molar percentage of ß-1,4-galactosylated branches and the extent of α-1,3-galactosylated glycans (Figure 5). The importance of that finding from a biotechnological perspective is discussed below.

Discussion

NS0 cells are known to add the antigen Galα1-3Gal to N-glycans of therapeutic MAbs produced (**7**, **8**). Although no evidence yet shows that the presence of that antigen on IgG is immunogenic to humans, regulatory agencies might express concerns about Galα1-3Gal containing therapeutic glycoproteins (**8**). In an effort to reduce the epitope from NS0-produced Abs, we analyzed and studied glycosylation profiles of MAbs produced from different NS0 clones using a new method developed by Callewaert called DSA-FACE (**11**): DNA-sequencer–aided fluorophore-assisted carbohydrate electrophoresis. We wanted to evaluate the sensitivity, isomer resolution, and quantification of the method while directly examining culture media samples to see whether terminal Galα1-3Gal varies among different clones.

To study how sensitive and quantitative the DSA-FACE method is, we performed a serial dilution of a purified antibody. The results (Figure 4) indicate that as little as 250 ng was enough to detect a general profile of glycosylation. To quantify less than 2% of the Galα1-3Gal content, however, about 2 mg of the antibody would be needed. That amount is compatible with high-throughput screening of Ab-secreting cultures in 96-well microplate format.

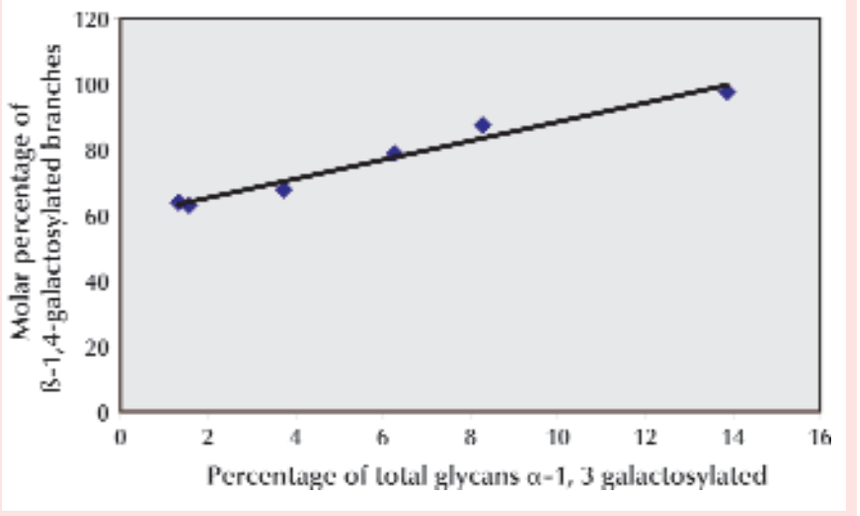
As with traditional CE methods, DSA-FACE can resolve isomers (Figure 3) and quantify glycoprofiles. Unlike CE, however, it is much more sensitive and offers higher throughput. As a result, DSA-FACE can not only differentiate glycoisomers, but quantify glycoprofiles at high throughput and high sensitivity. All that makes screening a large number of NS0 clones or process samples to obtain the best clone or the most optimal process much simpler and faster than was possible before. MS-based methods can be used to screen for low Galα1-3Gal, but as mentioned they are more complicated and less quantitative because additional enzymatic treatments are required to analyze isomer structures in N-linked glycosylation.

By analyzing glycoprofiles of the same MAb produced from different NS0 clones, we noticed that the degree of Galα1-3Gal content in an antibody varied between clones (Table 1). This is interesting because a published method to lower the Galα1-3Gal content of an NS0-produced therapeutic MAb relied on some kind of metabolic change to a single NS0 clone, such as media changes during fermentation optimization (**10**). If different NS0 clones can display varying antigen contents because of their genetic make-up, a simple screening for a clone that inherently produces low Galα1-3Gal can be more attractive. Not only are media and fermentation optimization time-consuming and difficult, but one optimal process condition for a

clone cannot be easily translated to another, so each different clone must be reoptimized.

We observed a strict linear correlation between the level of ß-1,4-galactosylation of recombinant Abs from NS0 cells and the level of α-1,3-Gal substitutents on those galactosylated branches. Low levels of α-1,3-galactosylation seem to be linked intricately to low levels of ß-1,4-galactosylation as well, due to a substrate–product relation. That should not cause concerns for therapeutic MAbs that function as neutralizing antibodies, because slight undergalactosylation should not affect their intended biological functions. But it may cause concern for MAbs that require effector functions (**1**) — such as Campath brand (alemtuzumab) from ILEX Pharmaceuticals (www.ilexonc.com) and Berlex Laboratories (www.berlex.com). In such cases, the relation between low levels of α-1,3-galactosylation and high levels of ß-1,4-galactosylation must be carefully balanced by screening for a desirable production clone or optimizing culture conditions and monitoring large-scale cell culture for consistency. That further emphasizes the need for good, reliable analytical tools, such as the DNA-sequencer–based tool we describe here, for studying both glycan modifications.

Figure 5: Relation galactosylated branches to extent of α-1,3-galctosylation (data calculated after sialidase digestion).



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