Chapter 4:
High-throughput glycosylation analysis of therapeutic immunoglobulin G by capillary gel electrophoresis using a DNA analyzer

Dietmar Reusch,¹ Markus Haberger,¹ Tobias Kailich,¹ Anna-Katharina Heidenreich,¹ Michael Kampe,¹ Patrick Bulau,¹ and Manfred Wuhrer²,³

¹ Pharma Biotech Development Penzberg, Roche Diagnostics GmbH, 82377 Penzberg, Germany
² Center for Proteomics and Metabolomics, Leiden University Medical Center, Leiden, The Netherlands
³ Division of BioAnalytical Chemistry, Department of Chemistry and Pharmaceutical Sciences, VU University Amsterdam, Amsterdam, The Netherlands

MAbs 2014; 6:185-96
Abstract

The Fc glycosylation of therapeutic antibodies is crucial for their effector functions and their behavior in pharmacokinetics and pharmacodynamics. To monitor the Fc glycosylation in bioprocess development and characterization, high-throughput techniques for glycosylation analysis are needed. Here, we describe the development of a largely automated high-throughput glycosylation profiling method with multiplexing capillary-gel-electrophoresis (CGE) with laser induced fluorescence (LIF) detection using a DNA analyzer. After PNGaseF digestion, the released glycans were labeled with 9-aminopyrene-1,3,6-trisulfonic acid (APTS) in 96-well plates, which was followed by the simultaneous analysis of up to 48 samples. The peak assignment was conducted by HILIC-UPLC-MS/MS of the APTS-labeled glycans combined with peak fractionation and subsequent CGE-LIF analysis of the MS-characterized fractions. Quantitative data evaluation of the various IgG glycans was carried out automatically using an in-house developed software solution. The excellent method accuracy and repeatability of the test system was verified by comparison with two UPLC-based methods for glycan analysis. Finally, the practical value of the developed method was demonstrated by analyzing the antibody glycosylation profiles from fermentation broths after small scale protein A purification.
Introduction

Recombinant monoclonal antibodies (mAbs) are valuable therapeutics for various conditions, including inflammatory diseases and cancer.\(^1\)\(^2\) The production of these glycoproteins in mammalian cell culture systems results in a remarkable macro- and microheterogeneity. The heterogeneity is often due to post-translational modifications like N-terminal pyroglutamic acid, C-terminal lysine heterogeneity, and glycosylation. Glycosylation is one of the most important post-translational modifications of mAbs because it affects properties critical to their development as therapeutics, e.g., solubility, structural stability, clinical efficacy.\(^3\)\(^4\)

Pronounced heterogeneity of protein glycoforms is often observed. Therapeutic mAbs are normally of the IgG isotype. Each heavy chain has in the Fc part an N-glycan chain linked to asparagine 297.\(^5\) Depending on the expression system, various complex-type, hybrid-type and high-mannose type glycans may be found at this site.\(^5\)\(^6\) The complex-type glycan species often show heterogeneity with regard to the presence of a core fucose and bisecting N-acetylgalcosamine. Additionally, they vary in galactose and sialic acid content of the antennae.\(^4\)\(^7\) IgGs may carry an additional N-glycan in the variable region of the fragment antigen-binding (Fab) portion. It has been shown that the Fab N-glycans are generally highly galactosylated and have a higher degree of sialylation.\(^8\)\(^9\)

Antibody effector functions are greatly influenced by the glycosylation pattern. The different glycan species often represent so-called critical quality attributes (CQAs) and must be carefully monitored.\(^10\) Galactosylation can affect the complement-dependent cytotoxicity by improving the binding to C1q.\(^11\) The absence of core fucose results in an increase in antibody-dependent cell-mediated cytotoxicity (ADCC).\(^12\)\(^13\) Sialylation may induce anti-inflammatory effects via Th2 signaling and decrease ADCC via reduced interaction with Fc gamma receptors.\(^14\)\(^15\) MAb glycosylation may also influence pharmacodynamic and pharmacokinetic behavior\(^16\)\(^-20\) and may even be the target of adverse immune reactions against terminal alpha1-3 bound galactose and N-glycolynueraminic acid.\(^21\)\(^-24\)

It is difficult to predict and control glycosylation patterns because N-glycan structures also depend on cultivation conditions, such as temperature, pH, and by-products like lactate.\(^25\) Therefore, monitoring IgG glycosylation is essential in the biotechnology industry and glycosylation is an important marker for process robustness in process development, medium development, clone selection, process characterization/validation studies and release analytics.\(^3\)\(^5\)\(^12\)

A wide range of analytical methods are available to monitor protein glycosylation targeting intact glycoproteins, glycopeptides (after proteolytic cleavage) or released glycans, including mass spectrometry (MS)-based methods, high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD), and high performance liquid chromatography (HPLC)-based methods relying on fluorescence labeling of the glycans.\(^3\)\(^26\) These methods have been reviewed by Huhn et al. and Marino et al.\(^27\)\(^28\) Capillary electrophoresis (CE) techniques have also been used for glycosylation analysis. In this approach, the glycans are generally derivatized with a fluorophore that carries one or more negative charges to allow electrophoretic separation. The tag, in most cases 9-
aminopyrene-1,3,6-trisulfonic acid (APTS) is introduced at the reducing terminus of the glycan via reductive amination \(^{29-31}\) and mass spectrometric detection may be applied.\(^ {32}\) Callewaert et al. introduced the electrophoretic separation and fluorescence detection of glycans by means of a multicapillary DNA analyzer.\(^ {33, 34}\) These systems, which are commonly used in molecular biology laboratories, use polyacrylamide-based gels for separation and apply an argon laser to excite the fluorescent tag of the glycans. The suitability of the method for glycosylation analysis was demonstrated by several investigators for a broad range of analytes, including biopharmaceuticals and clinical samples.\(^ {31, 35-41}\)

This approach is particularly amenable for high-throughput analysis of glycans, as DNA analyzers are available with various formats of capillary arrays, allowing the parallel measurement of up to 96 samples.\(^ {34}\) We present here the development of a high-throughput CGE-LIF method for IgG glycosylation analysis with a commercially-available DNA analyzer. For peak identification, APTS-labeled glycans were subjected to two-dimensional separation applying in the first dimension HILIC-UPLC with MS detection and in the second dimension CGE-LIF of the MS-characterized HILIC-UPLC peaks. In addition, spiking with commercially-available standards was applied for peak identification. The successful application of the CGE-LIF method in process development studies was demonstrated.
Figure 1. Schematic overview of experimental workflow (left) and workflow for identification of glycan structures (right).
Table 1. Structures, Summary of identification results

<table>
<thead>
<tr>
<th>Name and composition</th>
<th>Migration position [BPU] CGE-LIF</th>
<th>Retention time HILIC-HPLC of APTS-labelled glycans [min]</th>
<th>Expected monoisotopic molecular mass [Da] of z=2 charge state of APTS-labelled glycan</th>
<th>Experimental monoisotopic molecular mass [Da] of z=2 charge state of APTS-labelled glycan</th>
</tr>
</thead>
<tbody>
<tr>
<td>G0F H3N4F1</td>
<td></td>
<td>282</td>
<td>27.3</td>
<td>950.75</td>
</tr>
<tr>
<td>G1F H4N4F1</td>
<td></td>
<td>319/328*</td>
<td>30.2/31.1*</td>
<td>1031.78</td>
</tr>
<tr>
<td>G2F H5N4F1</td>
<td></td>
<td>363</td>
<td>33.8</td>
<td>1112.81</td>
</tr>
<tr>
<td>G0F-N H3N3F1</td>
<td></td>
<td>248</td>
<td>26.4</td>
<td>849.21</td>
</tr>
<tr>
<td>M5 H5N2</td>
<td></td>
<td>248</td>
<td>32.4</td>
<td>836.70</td>
</tr>
<tr>
<td>G0 H3N4</td>
<td></td>
<td>252</td>
<td>25.9</td>
<td>877.73</td>
</tr>
<tr>
<td>G1 H4N4</td>
<td></td>
<td>270/295*</td>
<td>29.0/29.9*</td>
<td>958.75</td>
</tr>
<tr>
<td>G2 H5N4</td>
<td></td>
<td>333</td>
<td>32.6</td>
<td>1039.78</td>
</tr>
</tbody>
</table>

* as G1F and G1 exist in two different isomers that can be separated with both methods two values are given
Figure 2. CGE-LIF electropherogram (in black) with basepairs used for calibration (in red) (A); HILIC-UPLC of APTS-labeled glycans used for fractionation of peaks and for quantitation (B); HILIC-UPLC of AB-labeled glycans used for comparison (C).

Results

Method development

A CGE-LIF method was established for the glycosylation analysis of therapeutic mAbs for two applications: (A) the glycosylation analysis at the drug substance level, and (B) the glycosylation analysis after automated small scale purification of the antibodies with protein A columns directly from fermentation supernatants. The method involves desalting of the IgG samples by ultrafiltration, glycan release by PNGase F, APTS labeling, glycan separation by CGE-LIF using a DNA analyzer, and automated data evaluation (Figure1A).

A typical CGE-LIF profile for the APTS-labeled glycans of MAb1, the formulated drug substance of a marketed therapeutic antibody, including the basepair standard as internal reference for migration times, is shown in Figure 2A. For comparison, the corresponding HILIC-UPLC profiles obtained after APTS labeling and 2-AB labeling are shown in Figure 2B and 2C, respectively. The profiles show high similarity, with smaller glycans eluting first in both CGE-LIF and HILIC-UPLC.

For the CGE-LIF separation, migration positions of glycan peaks were expressed in basepair units relying on a fluorescently-labeled DNA ladder that was detected by excitation at 587 nm and emission...
at 607 nm, not interfering with the detection of APTS-labeled glycans that occurs at 473 nm excitation and 520 nm emission.

CGE-LIF peak assignment on a DNA-analyzer is not feasible via direct coupling to a mass spectrometer, nor is the collection of eluting APTS-labeled glycans possible on such a system. Therefore, we relied on the two approaches for peak assignment in CGE-LIF (Figure 1B): 1) HILIC-UPLC with online mass spectrometric analysis, fractionation and subsequent CGE-LIF analysis of the collected APTS-labeled glycans that were assigned on the basis of the mass spectrometric data; and 2) CGE-LIF peak assignment via spiking experiments with commercially-available glycan standards that were APTS-labeled.

Figure 3. HILIC-UPLC-MS of APTS-labeled N-glycan pool from MAb1. (A) Total ion chromatogram (TIC). The peak indicated by an arrow corresponds to G0-F (H5N2) with retention time of 25.9 min. Inset: zoomed view of the [M-2H]2- ions. (B) MS-MS spectrum in negative ion mode, detected masses are [M-2H]2- ions.
CGE-LIF peak assignment via spiking with APTS-labeled glycans after HILIC-UPLC fractionation

Enzymatically released glycans of MAb1 were APTS-labeled and separated by HILIC-UPLC. For quantitation, peaks were detected with a fluorescence detector and the outlet of the column was online coupled to an ESI mass spectrometer. The fluorescence chromatogram is shown in Figure 2B. The corresponding total ion current (TIC) chromatogram is shown in Figure 3A. The identification of the HILIC-UPLC peaks was accomplished by comparing the experimentally detected molecular masses of the APTS-labeled glycans with their theoretical masses (Table 1). Because we used an Orbitrap mass spectrometer with a high mass accuracy, all abundant experimental masses could be unambiguously assigned to the corresponding glycan structure. As an example, the mass spectrum of the double-charged species of G0-F (H3N4) is shown in Figure 3B. Moreover, all detectable glycan structures were confirmed by MS/MS experiments as shown, for example, by the MS/MS spectrum of G0 (H3N4) (Figure 3). The fragmentation was conducted in the negative ion mode. Predominantly [M-2H]^2- ions of the y-type were observed. This might be due to the negative charge of the APTS label. Only minor signals corresponding to ring fragments could be detected, and the loss of hexoses and n-acetyl-hexose could be detected, and hence the structure unambiguously assigned.

The two isomeric structures of G1F (H4N4F1) corresponding to the man alpha 1,3- and the alpha 1,6-arm could be separated with HILIC-UPLC and CGE-LIF, but could not be unambiguously assigned with MS/MS. The same holds true for the two isomeric structures of G1 (H4N4); however, literature suggests that the earlier eluting peaks correspond to the alpha 1,6-arm and the later eluting peak to the alpha 1,3 arm. The relative amount of the peaks is consistent with that reported for IgG1 glycan structures. Because the effect of the two isomeric structures is unclear, we report the sum of G1F (H4N4F1) and G1 (H4N4), respectively.

From another HILIC-UPLC run, fractions of the APTS-labeled glycans of MAb1 were collected and individual fractions corresponding in each case to a peak in HILIC-UPLC were spiked into the APTS-labeled glycans of MAb1 prior to CGE-LIF analysis. Figure 4A shows the CGE-LIF electropherogram of APTS-labeled glycans of MAb1, Figure 4B and 4C show the CGE-LIF results of APT- labeled glycans of MAb1 spiked with the collected peak assigned by mass spectrometric analysis as G0 (H3N4) or M5 (H5N2), respectively. The first spiking experiment identified G0 (H3N4) as the minor peak eluting at 252 base pair units (Fig4B). In the second experiment M5 (H5N2) was spiked into the APTS-labeled glycans of MAb1 showing co-elution with the peak at 248 base pair units. The same holds true when g0F-N (H3N3F1) is spiked into the APTS-labeled glycans of MAb1 (Figure 4D). It could thus be shown that G0 (H3N4) could be unambiguously assigned in the CGE-LIF glycan pattern, whereas M5 (H5N2) and G0F-N (H3N3F1) co-elute. With the exception of these co-eluting glycan structures all abundant peaks of the CGE-LIF electropherogram could be assigned by applying this strategy (summarized in Table 1).
Figure 4. Spiking of HILIC-UPLC separated APTS-labeled glycans into CGE-LIF glycan profile. (A) CGE-LIF electropherogram of MAb1 glycan pool without spiking (B) CGE-LIF electropherogram of MAb1 glycan pool with spiking of G0 (H3N4) (C) CGE-LIF electropherogram of MAb1 glycan pool with spiking of M5 (H5N2).

CGE-LIF peak assignment via spiking with commercially-available glycan standards

To confirm the assignment of the peaks of the CGE-LIF glycan pattern and to corroborate the co-elution of M5 (H5N2) and G0F-N (H3N3F1), commercially-available glycan standards were APTS-labeled and spiked into the glycans of MAb1 after APTS-labeling and subsequently analyzed with CGE-LIF.

As an example, the spiking of MAb1 with APTS-labeled G0 (H3N4), M5 (H5N2) and G0F-N (H3N3F1) is shown in comparison to the non-substituted MAb1 sample in Figure 5.

These experiments confirm the results achieved via spiking with APTS-labeled glycans after HILIC-UPLC fractionation and demonstrate that the two glycan species M5 (H5N2) and G0F-N (H3N3F1) cannot be separated by the applied CGE-LIF system. This must be taken into account if, for example, if the amount of afucosylation of the therapeutic antibody has to be determined, as this peak may contain both a core-fucosylated (G0F-N) and a non-core-fucosylated glycan species (M5). With HILIC-UPLC analysis of APTS-labeled and 2-AB-labeled glycans of MAb1, we could elucidate that the selected material does contain both G0F-N and M5 structures amounting to 0.7% and 1.7% with HILIC-UPLC of APTS-labeled glycans and 0.5% and 2.0% with HILIC-UPLC of 2-AB-labeled glycans.
glycans, respectively (Table 2). All major glycan peaks could be assigned with the employed strategy (Table 1, raw data not shown).

In summary, the main species detected were G0F (H3N4F1), G1F (H4N4F1) and G2F (H5N4F1) with a relative abundance of 36.6%, 43.3% and 8.7% and migration positions of 282, 319, and 363 base pair units (Table 1 and 2). Minor components, however, could also be detected at migration positions ranging from 248 to 333 base pair units. These are G0 (H3N4) with a relative abundance of 4.8%, G1 (H4N4) with a relative abundance of 3.2% and G2 (H5N4) with a relative abundance of 0.4%. As previously stated, the glycan species G0F-N (H3N3F1) and M5 (H5N2) co-elute at 248 basepairs so they can only be reported as sum, with a relative abundance of 1.8% (see Tables 1 and 2).

Figure 5. Spiking of APTS-labeled commercially-available reference glycans into CGE-LIF glycan profile. (A) CGE-LIF electropherogram of MAb1glycan pool without spiking (B) CGE-LIF electropherogram of MAb1glycan pool with spiking of M5 (H5N2) (C) CGE-LIF electropherogram of MAb1 with spiking of G0F-N (H3N3F1).
Repeatability of the CGE-LIF method

To test the repeatability of the developed CGE-LIF method, eight individual samples of MAb1 were deglycosylated and the glycans were labeled separately with APTS and subsequently analyzed with CGE-LIF by the DNA analyzer. The analyses were repeated twice on different days. As can be seen in Figure 6, the repeatability (given in the standard deviation of the individual bars) and the intermediate precision (given in the difference between the individual bars in different color for each glycan species) was excellent. The relative standard deviation for the major 4 peaks was found to be below 0.5% throughout. In conclusion, our glycan analysis method is very robust and precise.

Figure 6. Repeatability of the IgG glycosylation analysis by CGE-LIF. IgG samples were analyzed in eightfold on three different days. Released glycans were analyzed by CGE-LIF after APTS-labeling, peaks were picked and assigned, and peak areas were normalized. Relative peak areas are given, and standard deviations are indicated.
**Sensitivity of the CGE-LIF method**

All glycans expected to be present in the Fc part of an antibody expressed in Chinese hamster ovary (CHO) cells could be detected with a sensitivity that is comparable to the sensitivity achieved with chromatographic methods. The absolute sensitivity of the CGE-LIF method, however, is much higher. We start with 5 µg IgG (~ 33 pmol). Because a 1:20 dilution step is included in the sample preparation, it should also be possible to analyze 0.25 µg IgG corresponding to 1.6 pmol. The limit of detection is surely far below as we could also detect and quantitate peaks with a relative abundance of 0.4%.

**Comparison to other methods for glycosylation analysis**

The developed method was used to analyze IgG glycosylation of MAb1. For comparison, two other methods for glycosylation analysis were applied. In the first approach, glycans of MAb1 were cleaved with PNGase F, APTS-labeled, separated with a HILIC-UPLC and analyzed with a fluorescence detector.

The second method, HILIC-UPLC of 2-AB labeled glycans, is the “gold standard” established in our laboratory. Briefly, glycans were released with PNGase F, labeled with 2-amino benzamide, cleaned up with a Nanosep centrifugal device and separated with HILIC-UPLC with fluorescence detection. For better comparison of all methods, six replicates were analyzed and the standard deviations were calculated.

Besides the main glycoforms that are expected for a therapeutic antibody produced in CHO cells, which are H3N4F1 (G0F), H4N4F1 (G1F) and H5N4F1 (G2F) for all applied methods, the same minor glycan species could be detected and quantified as well, with standard deviations between 0.1% and 0.8%, which signify a high precision and repeatability (Table 2). Because G0F-N (H3N3F1) and M% (H5N2) cannot be separated by CGE-LIF, the sum value is given under M5. The relative amounts of MAb1 N-glycans as determined by the three methods were very similar, although different labels and separation techniques were employed.

In conclusion, our newly developed method gives essentially the same results as our gold standard method that we have validated for glycosylation analysis of biotherapeutics.
Table 2. Quantitation, comparison to alternative methods (where applicable standard deviations are
given in parentheses. Six replicates were analyzed side by side).

<table>
<thead>
<tr>
<th>Name and composition</th>
<th>Structure</th>
<th>Relative amount (%) determined with CGE-LIF</th>
<th>Relative amount (%) determined with HILIC-UPLC of APTS-labeled glycans</th>
<th>Relative amount (%) determined with HILIC-UPLC of AB-labeled glycans</th>
</tr>
</thead>
<tbody>
<tr>
<td>G0F H3N4F1</td>
<td></td>
<td>36.6 (0.4)</td>
<td>35.5 (0.5)</td>
<td>34.7 (0.8)</td>
</tr>
<tr>
<td>G1F H4N4F1</td>
<td></td>
<td>43.3 (0.5)*</td>
<td>43.6 (0.6)</td>
<td>45.2 (0.4)</td>
</tr>
<tr>
<td>G2F H5N4F1</td>
<td></td>
<td>8.7 (0.1)</td>
<td>9.1 (0.0)</td>
<td>10.2 (0.5)</td>
</tr>
<tr>
<td>G0F-N H3N3F1</td>
<td></td>
<td>co-elutes with H5N2</td>
<td>0.7 (0.1)</td>
<td>0.5 (0.1)</td>
</tr>
<tr>
<td>M5 H5N2</td>
<td></td>
<td>1.8 (0.1)**</td>
<td>1.7 (0.1)</td>
<td>2.0 (0.2)</td>
</tr>
<tr>
<td>G0 H3N4</td>
<td></td>
<td>4.8 (0.1)</td>
<td>4.7 (0.1)</td>
<td>4.1 (0.4)</td>
</tr>
<tr>
<td>G1 H4N4</td>
<td></td>
<td>3.2 (0.2)*</td>
<td>2.8 (0.2)</td>
<td>3.3 (0.2)</td>
</tr>
<tr>
<td>G2 H5N4</td>
<td></td>
<td>0.4 (0.1)</td>
<td>0.5 (0.0)</td>
<td>0.3 (0.1)</td>
</tr>
</tbody>
</table>

* The sum of the relative amounts of the two isomeric structures is given

** Because G0F-N and M5 co-elute, the sum of the relative amounts is given
Application for process development

One of the typical applications for the developed test method is analysis of the glycosylation profile of a fermentation time-course. In this case, samples were taken each day from the beginning of the fermentation to day 10, purified by protein A chromatography, and analyzed with CGE-LIF after glycan release and labeling. Glycan analysis was performed in a single experiment after finalization of the fermentation.

The time-course for six different glycoforms from day 1 to day 10 is shown in Figure 7. As expected for a fermentation of therapeutic antibodies in CHO cells, the relative amount of G0F glycan (H3N4F1) increases with time and, accordingly, G1F glycan (H4N4F1) decreases with time. For the other glycans, the difference with fermentation time is less pronounced.25

![Figure 7. Glycosylation time-course of a fermentation experiment.](image)

Discussion

The biological relevance of glycosylation and the state of the art analytical glycan profiling methods have been reviewed by several authors43-45. Recently also high throughput technologies for glycan analysis have been reported46-48. We developed a high-throughput method for the glycosylation analysis of recombinantly expressed IgGs by capillary gel electrophoresis using a DNA analyzer. The high-throughput analysis is achieved by a high degree of automation and by multiplexing that allows up to 96 samples to be analyzed concurrently. We used a commercially-available labeling kit from

<table>
<thead>
<tr>
<th>Name and composition</th>
<th>Structure</th>
<th>Relative amount (%) determined with CGE-LIF</th>
<th>Relative amount (%) determined with HILIC-UPLC of APTS-labeled glycans</th>
<th>Relative amount (%) determined with HILIC-UPLC of AB-labeled glycans</th>
</tr>
</thead>
<tbody>
<tr>
<td>G0F</td>
<td>H3N4F1</td>
<td>36.6 (0.4)</td>
<td>35.5 (0.5)</td>
<td>34.7 (0.8)</td>
</tr>
<tr>
<td>G1F</td>
<td>H4N4F1</td>
<td>43.3 (0.5)*</td>
<td>43.6 (0.6)</td>
<td>45.2 (0.4)</td>
</tr>
<tr>
<td>G2F</td>
<td>H5N4F1</td>
<td>8.7 (0.1)</td>
<td>9.1 (0.0)</td>
<td>10.2 (0.5)</td>
</tr>
<tr>
<td>G0F-N</td>
<td>H3N3F1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.7 (0.1)</td>
<td>0.5 (0.1)</td>
<td></td>
</tr>
<tr>
<td>M5</td>
<td>H5N2</td>
<td>1.8 (0.1)**</td>
<td>1.7 (0.1)</td>
<td>2.0 (0.2)</td>
</tr>
<tr>
<td>G0H3N4</td>
<td></td>
<td>4.8 (0.1)</td>
<td>4.7 (0.1)</td>
<td>4.1 (0.4)</td>
</tr>
<tr>
<td>G1H4N4</td>
<td></td>
<td>3.2 (0.2)*</td>
<td>2.8 (0.2)</td>
<td>3.3 (0.2)</td>
</tr>
<tr>
<td>G2H5N4</td>
<td></td>
<td>0.4 (0.1)</td>
<td>0.5 (0.0)</td>
<td>0.3 (0.1)</td>
</tr>
</tbody>
</table>

* The sum of the relative amounts of the two is given

** Because G0F-N and M5 co-elute, the sum of the relative amounts is given
Prozyme that was originally developed for conventional capillary electrophoresis applications. This helps to minimize the hands-on time in the laboratory. The theoretical sample throughput is enormous. Using the 96-capillary setting, and allowing for the 45 min run-time for one CGE-LIF analysis, up to 3000 samples can be analyzed in a day.

As the DNA analyzer cannot be directly coupled to a mass spectrometer, alternative methods for peak characterization must be employed. We used a pre-fractionation approach with concomitant MS/MS characterization of APTS-labeled glycans. As an alternative method, commercially-available standards were used in spiking experiments. The two presented strategies for the characterization of the glycans should be applicable for the glycan analysis of mAbs from various expression systems, as well as other therapeutic glycoproteins. Glycan structural assignment has to be conducted only once for an individual antibody because the characterized peaks can be linked directly to basepair units in the developed software, and hence the peaks can be unambiguously assigned in each run.

The CGE-LIF DNA analyzer system has a separation power comparable to HILIC-UPLC after APTS-labeling and 2-AB labeling, respectively. The entire glycan repertoire of MAb1, which is typical for a therapeutic antibody expressed in CHO cells, could be separated with the exception of G0F-N (H3N3F1) and M5 (H5N2), which were found to co-elute. If an exact level of afucosylation must be calculated, this has to be taken into account as G0F-N (H3N3F1) is fucosylated and M5 (H5N2) is not. The detected relative amounts of MAb1 glycan species were highly comparable to the results we obtained with our “gold standard” method HILIC-UPLC following 2-AB-labeling or APTS-labeling. The method proved to be very robust. We used 4 different batches of capillaries and at least 5 different batches of running buffer. As our laboratories are temperature-controlled, we do not know to which extent the separation is influenced by temperature changes.

With the experiments described here, we could show that the CGE-LIF method has an excellent separation power, a very good repeatability and intermediate precision, and possesses a high robustness. The standard deviations were very close to those reported previously by Schwarzer et al. The use of DNA fragments for normalization was reported by other groups and proved to be very useful in our hands.\textsuperscript{40} The comparison to other methods gives us also a very high confidence that we determine ratios of glycan species without a vast bias. Over the course of two years, we analyzed thousands of samples and the CGE-LIF with a DNA analyzer is now used routinely for samples for process development. We are currently developing adapted CGE-LIF methods for the analysis of glycoengineered antibodies, Fab glycosylated antibodies and other glycoproteins like EPO.

The CGE-LIF method can be used with fermentation broths. In this case, a small scale purification with protein A material must also be employed, but this step can be automated. We presented the glycosylation data of a fermentation time-course used for process development, process characterization and process validation studies. The change of the glycosylation status of an IgG in the course of a fermentation could be shown. Likewise, the method should be capable of detecting glycosylation changes associated with process changes.
In conclusion, CGE-LIF analysis with a DNA analyzer can generate data that help to keep a production process within the desired design space or assess that a comparable drug substance is being produced after process changes (comparability), but could be used for release analytics due to its robustness. Because the method is rather simple and does not rely on mass spectrometry, we expect that it can also be run in non-specialized laboratories or in an at-line analytics setting near the fermentation process. The method is fast, robust, and cost-efficient, and has great potential for use in all laboratories involved in glycomics.

**Materials and Methods**

MAb1, a marketed antibody, was produced in a CHO cell line and purified by the Downstream Processing Group at Roche GmbH. rProtein A-Sepharose™ fast flow product was obtained from GE Healthcare. N-Glycosidase and 10x phosphate buffered saline (PBS) were obtained from Roche Diagnostics. Water was purchased from J.T.Baker. Acetonitrile was bought from Fluka. Glycan standards were purchased from Prozyme. The sample preparation method was designed in 96-well plates allowing full automation. For automation of pipetting steps we used a Hamilton Microlab Star Robot.

**Small scale purification with Protein A columns**

1 ml of fermentation supernatant was filtered in AcroPrep™ Advance 96-Well Filter Plates 1.2 µm Supor from Pall and applied to an Atoll column filled with 50 µl MabSelect SuRe (GE Healthcare). MAb1 was eluted with 2.5 mM HCl pH2.6. The protein content was assessed with a UV spectrometer and 5 µg MAb1 were automatically transferred to a AcroPrep™ Advance 96-Well Filter Plates 30K Omega from Pall by means of a TECAN Freedom Evo robot. Water was added to give a final volume of 300 µl. The plates were centrifuged three times after addition of 300 µl water for five minutes with 1500 g.

**Sample preparation bulk samples**

Samples from small purification with Protein A columns or bulk samples with an IgG content of about 5 µg were transferred to AcroPrep™ Advance 96-Well Filter Plates 30K Omega from Pall and water was added to give a final volume of 300 µl. The plates were centrifuged three times after addition of 300 µl water for five minutes with 1500 g.

**Deglycosylation of IgGs**

Samples were reconstituted in 50 µl of water containing 1 µl of PGNaseF (250 U of enzyme were dissolved in 250 µl H₂O). Filter plates were sealed and the samples were directly incubated on the filter plates at 37°C overnight. The released glycans were separated from IgG via the filter plates by centrifugation for 5 min at 1500g into 96-well receiver plates (Prozyme). Samples were dried by vacuum centrifugation.
Labeling and purification of glycans
Labeling was performed with the APTS-labeling module for 96-well plates (Prozyme, #GP96NG-APTS), consisting of reductant solution, APTS solution and APTS catalyst solution). For 96 samples, typically 104 µl of reductant, 260 µl of APTS catalyst and 104 µl of APTS solution were mixed. Dried glycans were reconstituted in 4.5 µl of the prepared APTS-labeling master mix. 96-well receiver plates were then sealed and the labeling was performed under light protection for 4 h at 50°C. Clean up after labeling reaction was done with GlykoPrep clean up (CU) cartridges (Prozyme). 20 ml of 5x APTS sample loading buffer (Prozyme) was filled up to 100 ml with acetonitrile. Samples were then diluted in 200 µl APTS sample loading buffer with thorough mixing and subsequently loaded onto the CU cartridges using 3 min centrifugal force at 300 g followed by 1 min at 1000 g. Then CU cartridges were washed 2 times with 200 µl of APTS sample loading buffer by centrifugation for 3 min at 300 g to remove excess dye and labeling side products. Samples were then eluted with 2 times 50 µl of water by centrifugation for 3 min at 1000 g.

Sample preparation for CGE-LIF
For 96 samples, 1250 µl Hi-Di Formamide (Applied Biosystems product code 4311320) was mixed vigorously with 3.5 µl of Basepair Size Standard (Applied Biosystems 500 Rox Size Standard product code 401734). Cleaned up samples were diluted 1:20 with water. Prior to analysis, 2 µl of diluted samples were then mixed with 10 µl HI-DI Formamide Basepair Size Standard mixture.

CGE-LIF measurement of APTS-labeled glycans
For the analysis, we used a 48 capillary array of 50 cm length (Applied Biosystem filled with Pop-7™ Polymer (Applied Biosystems). Injection was performed with an injection voltage of 3 kV for 15 sec. Run time was set to 1800 s and run time voltage was set at 15 kV.

HILIC-UPLC of 2-AB labeled glycans
Glycans were released with PNGaseF, dried in a vacuum centrifuge, labeled with 2-aminobenzamide cleaned up with a NanoSep centrifugal device (Pall Life Sciences) and separated by HILIC-HPLC with fluorescence detection.49, 50.

HILIC-UPLC-MS/MS of APTS-labeled glycans
APTS-labeled glycans were prepared as described before, but we used 500 µg instead of 5 µg of Mab1 for HILIC-UPLC-MS/MS experiments. HILIC-UPLC analysis were performed using ACQUITY UPLC BEH Glycan column from Waters (2.1 x 150 mm, 1.7 µm) with constant flow of 500 µl/min using gradient elution from 25% to 40% of Eluent A in 38.5 min. Eluent A consisted of 50 mM ammonium formate at pH 4.4 in water and Eluent B was acetonitrile (J.T. Baker MS grade). Prior to analysis 25 µl of labeled glycans in water were diluted in 75 µl acetonitrile (J.T Baker MS grade). 35 µl of the diluted glycans were injected. The UPLC system was equipped with a fluorescence detector (APTS detection extinction 473 nm; emission 520 nm) and directly coupled to an Orbitrap Velos mass spectrometer (Thermo scientific). Full MS and MS/MS of APTS-labeled glycans were acquired online in one experiment. The MS was operated in the negative ion mode. Source settings
for MS were chosen as follows: capillary voltage was set to 5.0 kV; capillary temperature at 275°C; sheath gas flow at 42.0 (arb) and auxiliary gas flow at 5.0 (arb). For full MS we used analyzer FTMS (Fourier transform mass spectrometry) mode, scan type full, resolution at 30000 and data acquisition type profile. MS/MS experiments were conducted in the Ion Trap mode, collision energy was set fix at 35 mV and data acquisition type centroid. For fraction collection, fractions were collected manually.

CGE-LIF data analysis
The data analysis was performed by an in-house developed Matlab application. The software normalized the migration time on the internal base pair standard by an regression function of 2nd polynomial order. To eliminate minor deviations and to increase the comparability of different datasets, the peak corresponding to G0 (H3N4F1) was shifted to an bpu-value of 281.9. For the assignment of the remaining peaks, a database that contained the bpu-values and the accepted deviation of the expected glycans was used. Finally, the area of the assigned peaks was determined and the relative area of the glycans calculated.

Acknowledgements
We thank Jana Gassner, Bernd Maier and Stephanie Esslinger for excellent technical assistance.

M. Wuhrer was supported by the European Union's Seventh Framework Program (FP7-Health-F5-2011) under grant agreement n°278535 (HighGlycan).
References

Chapter 5:
Comparison of methods for the analysis of therapeutic immunoglobulin G Fc-glycosylation profiles – part 1: separation-based methods

Dietmar Reusch,¹ Markus Haberger,¹ Bernd Maier,¹ Maria Maier,¹ Ronny Kloseck,¹ Boris Zimmermann,¹ Michaela Hook,¹ Zoltan Szabo,² Samnang Tep,² Jo Wegstein,² Nadja Alt,¹ Patrick Bulau,¹ Manfred Wuhrer³,⁴

¹ Pharma Biotech Development Penzberg, Roche Diagnostics GmbH, 82377 Penzberg, Germany
² ProZyme, Inc., 3832 Bay Center Place, Hayward, CA 94545-3619, USA
³ Center for Proteomics and Metabolomics, Leiden University Medical Center, Leiden, The Netherlands
⁴ Division of BioAnalytical Chemistry, Department of Chemistry and Pharmaceutical Sciences, VU University Amsterdam, Amsterdam, The Netherlands

MAbs 2015; 7:167-79
Abstract
Immunoglobulin G (IgG) crystallizable fragment (Fc) glycosylation is crucial for antibody effector functions, such as antibody-dependent cell-mediated cytotoxicity, and for their pharmacokinetic and pharmacodynamics behavior. To monitor the Fc-glycosylation in bioprocess development, as well as product characterization and release analytics, reliable techniques for glycosylation analysis are needed. A wide range of analytical methods has found its way into these applications. In this study, a comprehensive comparison was performed of separation-based methods for Fc-glycosylation profiling of an IgG biopharmaceutical. A therapeutic antibody reference material was analyzed 6-fold on two different days, and the methods were compared for precision, accuracy, throughput and other features; special emphasis was placed on the detection of sialic acid-containing glycans. Seven, non-mass spectrometric methods were compared; the methods utilized liquid chromatography-based separation of fluorescent-labeled glycans, capillary electrophoresis-based separation of fluorescent-labeled glycans, or high-performance anion exchange chromatography with pulsed amperometric detection. Hydrophilic interaction liquid chromatography-ultra high performance liquid chromatography of 2-aminobenzamide (2-AB)-labeled glycans was used as a reference method. All of the methods showed excellent precision and accuracy; some differences were observed, particularly with regard to the detection and quantitation of minor glycan species, such as sialylated glycans.
Table 1. Overview of used methods.

<table>
<thead>
<tr>
<th>Method</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>HILIC(2-AB)</td>
<td>2-AB labeling of released glycans; separation with HILIC-UPLC</td>
</tr>
<tr>
<td>The Reference Method</td>
<td></td>
</tr>
<tr>
<td>HILIC(IAB)</td>
<td>Labeling of released glycans with InstantAB; separation with HILIC-HPLC</td>
</tr>
<tr>
<td>CE-LIF(APTS-HR1)</td>
<td>APTS-labeling of released glycans and separation with CE</td>
</tr>
<tr>
<td>DSA-FACE(APTS)</td>
<td>DSA-FACE employing APTS-labeling of released glycans, separation with multiplexing CGE-LIF</td>
</tr>
<tr>
<td>CE-LIF(APTS-HR2)</td>
<td>Labeling of released glycans with Rapid-Reductive-Amination APTS; separation with capillary electrophoresis</td>
</tr>
<tr>
<td>CCGE(ANTS)</td>
<td>ANTS-labeling of released glycans with Rapid-Reductive-Amination ANTS; separation with cartridge-based capillary gel electrophoresis</td>
</tr>
<tr>
<td>HPAEC-PAD</td>
<td>Separation with high pH anion exchange HPLC; detection with pulsed amperometric detection</td>
</tr>
</tbody>
</table>

Introduction

Recombinant monoclonal antibodies (mAbs) are efficacious therapeutic agents for various disease areas, including inflammatory and autoimmune diseases as well as cancer.¹, ² The use of mammalian expression systems results in a remarkable heterogeneity of mAb products, generally due to post-translational modifications, such as N- and C-terminal modifications, deamidation, isomerization and glycosylation. Glycosylation is a critical post-translational modification because it may affect mAbs characteristics such as solubility, stability, pharmacokinetic and pharmacodynamics properties, as well as in vivo efficacy.³, ⁴

Currently, therapeutic mAbs are almost exclusively the IgG isotype, and bear in their Fc domain an N-glycan chain linked to asparagine 297 (numbering according to Kabat).⁵ Depending on the cellular expression system, these N-glycans are a mixture of complex-, hybrid- or high mannose-type glycans. The main part is normally composed of fucosylated complex-type biantennary oligosaccharides that may lack a core fucose and may include a bisecting N-acetylgalactosamine. Additionally, they vary in galactose and sialic acid content.⁴, ⁶⁻⁸ Some therapeutic mAbs may carry additional N-glycans in the variable regions of the antigen-binding (Fab) domain and these glycans may affect antigen binding.⁹
The glycosylation pattern has a great impact on the antibody effector functions. A relatively high amount of galactose may result in activation of the complement system and, by IgG binding to C1q, results in complement-dependent cellular cytotoxicity.\textsuperscript{11} Afucosylation (lack of core fucose) promotes antibody-dependent cellular cytotoxicity (ADCC) by increased binding of the IgG Fc portion to FcγRIIIa on natural killer cells.\textsuperscript{12, 13} Glycoforms can also affect pharmacodynamics and pharmacokinetics. Recent studies described clear evidence for selective clearance of oligomannose species (high mannose-type) of Fc glycans.\textsuperscript{14, 15} Sialylation may induce anti-inflammatory effects via Th2 signaling and decrease ADCC via reduced interaction with Fcγ receptors.\textsuperscript{16, 17} Additionally, some IgG glycan structures such as α1,3-bound galactose and N-glycolyneuraminic acid may be involved in adverse immune reactions.\textsuperscript{18, 19} Due to its various functional implications, the glycosylation pattern of a therapeutic antibody may represent a critical quality attribute, and therefore may require close monitoring during bioprocess development and routine manufacturing.\textsuperscript{20} A wide range of state-of-the-art analytical methods to monitor Fc-glycosylation is available. In principle the methods can be sub-divided into three categories:\textsuperscript{3, 21-23} (1) Analysis of the IgG molecule with electrospray ionization mass spectrometry (ESI-MS) – either on the intact molecule after reduction of disulphide bonds, or after a limited digestion with a proteolytic enzyme and deduction of the overall glycan composition;\textsuperscript{24-26} (2) Enzymatic release of the Fc glycans and measurement with mass spectrometric methods, by HPLC with pulsed amperometric detection or by capillary electrophoresis (CE)/HPLC-based methods after fluorescent labeling;\textsuperscript{27, 28} and (3) Proteolytic cleavage of the IgG molecule and analysis of the glycopeptides with matrix-assisted laser desorption/ionization-mass spectrometry (MALDI-MS) or electrospray ionization-mass spectrometry ESI-MS.\textsuperscript{29-32} Comparisons of different methods for analysis of IgG Fc-glycosylation have been reported, but these studies included a limited number of methods or compared mainly mass spectrometry-based methods.\textsuperscript{33-38} Thus, a thorough comparison of different methods for glycoanalysis is still lacking. As a consequence, we performed an extensive study on both non-mass spectrometric and mass spectrometric methods for IgG Fc-glycosylation analysis. The study involved three laboratories: a biopharmaceutical company (Roche Diagnostics), an academic research laboratory (Leiden University Medical Center) and a vendor of tools for glycan analysis (ProZyme Inc.). The same mAb sample was analyzed 6-fold on two different days. Special attention was paid to the measurement of low levels of sialylation. Methods were compared with regard to separation power, precision, accuracy, required resources and throughput. Due to its wide acceptance, hydrophilic interaction liquid chromatography-ultra high performance liquid chromatography (HILIC-UHPLC) of N-glycans after labeling with 2-AB was used as the reference method against which the other methods were compared [HILIC(2-AB), the Reference Method].\textsuperscript{22} The entire study has been divided into two parts; the first part, comparing the non-mass spectrometric methods, is presented here.
Table 2. Quantitative evaluation of method performance. Each glycoanalytical method was applied in two series (batches), 6 replicates per batch. Relative abundance of the various glycan species are given in percent, with standard deviations in parentheses. For G1F(1,6), fucosylated, monogalactosylated biantennary glycan with galactosylation of the 1,6-arm, the percentage within the overall G1F species is given in brackets. Key: H, hexose; N, N-acetylhexosamine; F, deoxyhexose; S, N-acetylneuraminic acid (sialic acid); G0F-N, agalacosylated, core-fucosylated, monoantennary species, etc.; n.d.: not detected; n.a.: not applicable.

<table>
<thead>
<tr>
<th>Glycan species No.</th>
<th>Short name [composition]</th>
<th>Structural scheme</th>
<th>HILIC(2-AB) Reference Method</th>
<th>CE-LIF(APTS-HR1)</th>
<th>DSA-FACE(APTS)</th>
<th>CE-LIF(APTS-HR2)</th>
<th>CCGE(ANTS)</th>
<th>HPAEC-PAD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>G0F [H3N4F1]</td>
<td></td>
<td>35.5 (0.1)</td>
<td>35.3 (0.1)</td>
<td>33.5 (0.4)</td>
<td>34.1 (0.7)</td>
<td>36.1 (0.1)</td>
<td>36.0 (0.2)</td>
</tr>
<tr>
<td>2+3</td>
<td>G1F [H4N4F1]</td>
<td></td>
<td>43.4 (n.a.)</td>
<td>43.3 (n.a.)</td>
<td>42.8 (n.a.)</td>
<td>42.9 (n.a.)</td>
<td>45.3 (n.a.)</td>
<td>45.2 (n.a.)</td>
</tr>
<tr>
<td>2</td>
<td>G1F(1,6) [H4N4F1]</td>
<td></td>
<td>32.7 (0.1)</td>
<td>32.6 (0.1)</td>
<td>32.3 (0.1)</td>
<td>32.4 (0.2)</td>
<td>34.4 (&lt;0.1)</td>
<td>34.4 (0.1)</td>
</tr>
<tr>
<td>3</td>
<td>G1F(1,3) [H4N4F1]</td>
<td></td>
<td>10.8 (&lt;0.1)</td>
<td>10.7 (&lt;0.1)</td>
<td>10.6 (0.3)</td>
<td>10.5 (0.1)</td>
<td>10.9 (0.1)</td>
<td>10.7 (0.1)</td>
</tr>
<tr>
<td>4</td>
<td>G2F [H5N4F1]</td>
<td></td>
<td>9.5 (&lt;0.1)</td>
<td>9.6 (0.1)</td>
<td>10.6 (0.3)</td>
<td>10.2 (0.4)</td>
<td>9.4 (0.1)</td>
<td>9.4 (&lt;0.1)</td>
</tr>
<tr>
<td>Glycan species No.</td>
<td>Short name [composition]</td>
<td>Structural scheme</td>
<td>HILIC(2-AB) Reference Method</td>
<td>HILIC(IAB)</td>
<td>CE-LIF(APTS-HR1)</td>
<td>DSA-FACE(APTS)</td>
<td>CE-LIF(APTS-HR2)</td>
<td>CCGE(ANTS)</td>
</tr>
<tr>
<td>------------------</td>
<td>-------------------------</td>
<td>-------------------</td>
<td>-----------------------------</td>
<td>----------</td>
<td>------------------</td>
<td>----------------</td>
<td>------------------</td>
<td>------------</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1. series</td>
<td>2. series</td>
<td>1. series</td>
<td>2. series</td>
<td>1. series</td>
<td>2. series</td>
</tr>
<tr>
<td>5</td>
<td>G0 [H3N4]</td>
<td></td>
<td>4.6 (0.1)</td>
<td>4.7 (0.1)</td>
<td>4.5 (0.1)</td>
<td>3.7 (0.1)</td>
<td>4.1 (0.3)</td>
<td>4.7 (&lt;0.1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4.6 (0.1)</td>
<td>3.8 (&lt;0.1)</td>
<td>4.2 (0.2)</td>
<td></td>
<td></td>
<td>4.7 (&lt;0.1)</td>
</tr>
<tr>
<td>6+7</td>
<td>G1 [H4N4]</td>
<td></td>
<td>3.3 (n.a.)</td>
<td>3.4 (n.a.)</td>
<td>3.3 (n.a.)</td>
<td>2.4 (n.a.)</td>
<td>2.3 (n.a.)</td>
<td>2.9 (n.a.)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.6 (0.1)</td>
<td>1.4 (&lt;0.1)</td>
<td>1.6 (0.2)</td>
<td>1.8 (0.1)</td>
<td></td>
<td>1.8 (0.1)</td>
</tr>
<tr>
<td>6</td>
<td>G1 1,6 [H4N4]</td>
<td></td>
<td>2.4 (&lt;0.1)</td>
<td>2.4 (&lt;0.1)</td>
<td>2.4 (&lt;0.1)</td>
<td>1.6 (&lt;0.1)</td>
<td>2.0 (&lt;0.1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.9 (&lt;0.1)</td>
<td>0.9 (&lt;0.1)</td>
<td>0.9 (0.1)</td>
<td>0.8 (0.1)</td>
<td></td>
<td>0.7 (0.1)</td>
</tr>
<tr>
<td>7</td>
<td>G1 1,3 [H4N4]</td>
<td></td>
<td>0.3 (&lt;0.1)</td>
<td>0.4 (&lt;0.1)</td>
<td>0.3 (&lt;0.1)</td>
<td>n.d.</td>
<td>0.3 (&lt;0.1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.7 (&lt;0.1)</td>
<td>0.6 (&lt;0.1)</td>
<td>n.d.</td>
<td>0.4 (0.1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>G2 [H5N4]</td>
<td></td>
<td>0.5 (&lt;0.1)</td>
<td>0.5 (&lt;0.1)</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.5 (&lt;0.1)</td>
<td>0.5 (&lt;0.1)</td>
<td>n.d.</td>
<td>0.4 (&lt;0.1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>G0F-N [H3N3F1]</td>
<td></td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>0.4 (&lt;0.1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>G1F-N [H4N3F1]</td>
<td></td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>0.4 (&lt;0.1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>G0-N [H3N3]</td>
<td></td>
<td>0.4 (&lt;0.1)</td>
<td>0.4 (&lt;0.1)</td>
<td>0.2 (&lt;0.1)</td>
<td>0.3 (&lt;0.1)</td>
<td>0.4 (&lt;0.1)</td>
<td></td>
</tr>
<tr>
<td>Glycan species No.</td>
<td>Glycan name</td>
<td>Structural scheme</td>
<td>HILIC(2-AB)</td>
<td>HILIC(IAB)</td>
<td>CE-LIF(APTS-HR1)</td>
<td>CE-LIF(APTS-HR2)</td>
<td>CCEG(ANTS)</td>
<td>HPAEC-PAD</td>
</tr>
<tr>
<td>-------------------</td>
<td>-------------</td>
<td>-------------------</td>
<td>-------------</td>
<td>------------</td>
<td>------------------</td>
<td>------------------</td>
<td>------------</td>
<td>----------</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Reference Method</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1. series</td>
<td>2. series</td>
<td>1. series</td>
<td>2. series</td>
<td>1. series</td>
<td>2. series</td>
</tr>
<tr>
<td>12</td>
<td>M5 [H5N2]</td>
<td>![Structural diagram]</td>
<td>1.5</td>
<td>(&lt;0.1)</td>
<td>1.6</td>
<td>(&lt;0.1)</td>
<td>1.5</td>
<td>(&lt;0.1)</td>
</tr>
<tr>
<td>13</td>
<td>M6 [H6N2]</td>
<td>![Structural diagram]</td>
<td>0.1</td>
<td>(&lt;0.1)</td>
<td>0.1</td>
<td>(&lt;0.1)</td>
<td>0.2</td>
<td>(&lt;0.1)</td>
</tr>
<tr>
<td>14</td>
<td>G1F5 [H4N4F1S1]</td>
<td>![Structural diagram]</td>
<td>0.2</td>
<td>(&lt;0.1)</td>
<td>0.2</td>
<td>(&lt;0.1)</td>
<td>0.2</td>
<td>(&lt;0.1)</td>
</tr>
<tr>
<td>15</td>
<td>G2S1F [H5N4F1S1]</td>
<td>![Structural diagram]</td>
<td>0.7</td>
<td>(&lt;0.1)</td>
<td>0.7</td>
<td>(&lt;0.1)</td>
<td>1.2</td>
<td>(&lt;0.1)</td>
</tr>
<tr>
<td>16</td>
<td>G2S2F [H5N4F1S2]</td>
<td>![Structural diagram]</td>
<td>0.1</td>
<td>(&lt;0.1)</td>
<td>0.1</td>
<td>(&lt;0.1)</td>
<td>0.5</td>
<td>(&lt;0.1)</td>
</tr>
</tbody>
</table>
Results

In this study, seven non-mass spectrometric, separation-based methods (summarized in Table 1) were evaluated for the analysis of the Fc-glycosylation of an IgG1 monoclonal antibody (mAb1). One method relied on electrochemical detection of native, released glycans after separation at high pH: high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD). All other methods applied fluorescent labeling. Four methods used electrophoretic separation, which included conventional high-resolution capillary gel electrophoresis with laser-induced fluorescence [CE-LIF(APTS-HR1)], DNA-sequencer-aided fluorophore-assisted carbohydrate electrophoresis after 8-aminopyrene-1,3,6-trisulfonic acid (APTS) labeling for high-throughput screening [DSA-FACE(APTS)], high-resolution capillary gel electrophoresis with rapid labeling with APTS via reductive amination [CE-LIF(APTS-HR2)], and cartridge-based capillary gel electrophoresis with rapid 8-aminonaphthalene-1,3,6-trisulfonate (ANTS) labeling, in development specifically for screening [CCGE(ANTS)]. The CE methods differ with regard to the labeling method: APTS labeling for the “normal” method requires 4 to 24 h for labeling, whereas rapid reductive amination has been optimized to yield unbiased labeling in one hour. Another difference lies in the type of label used: three methods used APTS, and one ANTS. Different CE hardware systems were also used: CE-LIF(APTS-HR1) and CE-LIF(APTS-HR2) were analyzed on a Beckman Coulter 800 plus Pharmaceutical Analysis System; DSA-FACE(APTS) was analyzed on an Applied Biosystems ABI 3730xl DNA Analyzer; and CCGE(ANTS) was analyzed on ProZyme’s Merlin Cartridge-based Capillary Gel Electrophoresis System.

The two remaining chromatographic methods used HILIC separation of labeled glycans, with HILIC profiling of 2-AB-labeled glycans, one serving as the Reference Method and the other using InstantAB labeling [HILIC(IAB)]. Both were analyzed on a Waters ACQUITY UPLC® System (or a Dionex RSLC Ultimate 3000RS).

Three laboratories were involved in performing the experiments, an analytical laboratory in a development department, a quality control laboratory and a laboratory of a vendor of glycoanalytical tools.
Figure 1. HILIC-UPLC of 2-AB-labeled N-glycans [HILIC(2-AB), the Reference Method]. Key: blue square, N-acetylglucosamine; green circle, mannose; yellow circle, galactose; red triangle, fucose; purple diamond, N-acetylneuraminic acid.

Figure 2. HILIC-UPLC of N-glycans labeled with InstantAB [HILIC(IAB)]. For key, see Figure 1.
Figure 3. Capillary electrophoresis separation of APTS-labeled N-glycans with laser-induced fluorescence detection [CE-LIF(APTS-HR1)]. For key, see Figure 1.

Figure 4. Multiplexing CGE-LIF analysis of APTS-labeled N-glycans on a DNA sequencer [DNA-sequencer-aided fluorophore-assisted carbohydrate electrophoresis; DSA-FACE(APTS)]. For key, see Figure 1.
**Figure 5.** Capillary electrophoresis separation with laser-induced fluorescence detection of N-glycans labeled by rapid reductive amination [CE-LIF(APTS-HR2)]. For key, see Figure 1.

**Figure 6.** Capillary electrophoresis separation with laser-induced fluorescence detection of N-glycans labeled by rapid reductive amination [CCGE(Rapid Reductive Amination ANTS)]. For key, see Figure 1.
Figure 7. High-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) for the separation and detection of native N-glycans. For key, see Figure 1.

Peak assignment

Peak assignment of the two HILIC-based methods for analyzing glycans, [HILIC(2-AB) and HILIC(IAB)], was accomplished by online coupling of HILIC-UHPLC with ESI-MS. For isomeric glycan structures, as well as for confirmation of the mass spectrometric results, the elution position relative to a hexose homopolymer standard (glucose units) was taken into account for structural assignment. For HPAEC-PAD, peaks were identified by spiking commercially available glycan standards and by employing exoglycosidase digests. Additionally the peaks were confirmed by online desalting and coupling to ESI-MS. For CE-LIF(APTS-HR1), peaks were assigned by online coupling to ESI-MS as described by Gennaro et al. For DSA-FACE(APTS), assignment of peaks is described in Reusch et al. Briefly, glycan identification relied on the use of commercially available glycan standards (after APTS labeling) used to spike the DSA-FACE(APTS) analysis of APTS-labeled mAb1 glycans after HILIC-UHPLC fractionation. In addition, online ESI-MS/(MS) coupling of the HILIC-UHPLC separation of the APTS-labeled mAb1 N-glycans was employed for further structural elucidation.

The peak assignment for CE-LIF(APTS-HR2) and CCGE(ANTS) was accomplished by spiking commercially available glycan standards and by relying on the well-known order of elution of the different glycans.
Detected glycosylation features

All the methods facilitated separation of the main Fc N-glycan species that are typically found on therapeutic IgG mAbs produced in Chinese hamster ovary (CHO) cells (G0F, G1F, G2F, G0, G1 and M5; see Table 2 for key). The Reference Method allowed the resolution and quantitation of 15 glycan species (Figure 1). The related HILIC(IAB) method showed a similar resolution and likewise allowed the detection of 15 glycan structures (Figure 2).

The CE methods showed a total number of detected glycan species between 11 and 14 (Figures 3, 4, 5 and 6). HPAEC-PAD showed an excellent coverage of the mAb1 N-glycan species with 16 glycan species resolved and assigned. Remarkably, all seven methods allowed differentiation of isomers arising from upper (α1,6)- vs. lower (α1,3)-arm galactosylation of biantennary glycans, both with and without core-fucosylation [G1F(1,6); G1F(1,3); G1(1,6) and G1(1,3)]. The peak assignment of the monogalactosylated species was deduced from literature data: the peaks with upper (α1,6)-arm galactosylation were found for all methods to elute prior to the peaks with lower (α1,3)-arm galactosylation.61-66 Both HILIC-based methods and HPAEC-PAD also separated three sialic acid-containing glycans, namely G1FS [H4N4F1S1], G2S1F [H5N4F1S1] and G2S2F [H5N4F1S2]. All four CE-based methods separated two out of three of the sialylated species: CE-LIF(APTS-HR1), CE-LIF(APTS-HR2) and DSA-FACE(APTS) showed two resolved sialic acid-containing peaks (G1S1F and G2S2F; Figure 3, 4 and 5), while the G2S1F co-migrated with M5 and G0. CCGE(ANTS) detected G2S1F and G2S2F (Figure 6). The high-mannose structure M5 [H5N2] was separated and quantified with all methods, whereas M6 [H6N2] was only detected with the two HILIC-based methods and HPAEC-PAD. For mono-antennary structures (structures lacking an N-acetylglucosamine, such as G0F-N [H3N3F1], G1F-N [H4N3F1] and G0-N [H3N3] see Table 2), the separation capabilities were more diverse: G0F-N was separated with the two HILIC-based methods, with HPAEC-PAD and by CE-LIF(APTS-HR2), but not with CE-LIF(APTS-HR1) or CCGE(ANTS). Remarkably, G1F-N was only separated and identified by HPAEC-PAD (see Figure 7). The HILIC-based methods were able to separate nearly all species; only the minor species G1F-N [H4N3F1] co-eluted with G1(1,6) [H4N4]. Since G1F-N is generally found in CHO-derived mAbs at very low levels (<1%), this co-migration is regarded as being non-critical. In general, the glycan peaks are very well separated, resulting in excellent quantitation performance (Table 2). The G0-N structure was able to be separated with all methods except CCGE(ANTS). Other low-abundance glycan structures could also be separated. With the Reference Method, these minor structures were found, but for simplicity they were not included in Table 2: the mono-antennary structures G1-N [H4N3], M3 [H3N2] and M4 [H4N2]. HPAEC-PAD was the only method capable of separating all peaks included in the quantitation study. However, the baseline appears not to be as stable as for the HILIC- based method, impeding the peak integration for quantification.
Peak assignment for CE-based methods is more difficult because the instrument cannot easily be directly coupled to mass spectrometers without adapting the method. In addition, loading capacity of CE is relatively low, mainly due to low injection volumes, thereby complicating the coupling to mass spectrometric systems.

CE-LIF(APTS-HR1) and CE-LIF(APTS-HR2) were able to detect and quantify 11 peaks, but G2 and G0F-N could not be separated. DSA-FACE(APTS) separated 12 peaks that could be assigned, but some structures, G2S1F/G0 and M5/G0F-N, were found to be co-eluting. CCGE(ANTS) separated 11 peaks, missing G0F-N, G1F-N, G0-N, M6 and G1FS.

Method performance with regard to accuracy and precision

A summary of the quantitative methods evaluation is shown in Table 2. HILIC(2-AB), being our reference method, showed excellent precision with low standard deviations between each series (consisting of six replicates), and only minute differences in average relative abundance were observed between the two series analyzed on different days. The other methods tested, with the exception of the CE-LIF(APTS-HR1), showed a slightly greater difference between the mean results on different days. Nevertheless, the results obtained on different days were comparable, with the inter-day differences in relative intensities of all glycan species below 1% for all 7 methods. For the major glycan species, relative abundances determined with the various methods were in good agreement with values obtained by the Reference Method. The G0F species average 35.4% relative abundance for HILIC(2-AB), while this value was found to be slightly higher for CE-LIF(APTS-HR1), DSA-FACE(APTS) and HPAEC-PAD, ranging from 36.0% to 37.7%, and slightly lower for HILIC(IAB), CCGE(ANTS) and CE-LIF(APTS-HR2), ranging from 33.5% to 34.6%. The two isomeric G1F species were found with a combined average relative abundance of 43.4% with HILIC(2-AB). This value was again found to be slightly higher for CE-LIF(APTS-HR1), DSA-FACE(APTS) and HPAEC-PAD, ranging from 43.6% to 45.3%, and slightly lower for HILIC(IAB), CCGE(ANTS) and CE-LIF(APTS-HR2), ranging from 42.4% to 43.0%. The relative amounts of the upper (α1,6)- vs. lower (α1,3)-arm galactosylation was similar for all methods employed, with the portion of G1F(1,6) of the total G1F ranging from 74.4% to 77.7% for all 7 methods and two time points (Table 2). For HILIC(2-AB), the relative abundance of fucosylated mono-galactosylated species was 32.7% (α1,6) and 10.8% (α1,3), respectively, which was in good agreement with all other methods, ranging from 31.71% to 34.4% for the α1,6 variant, and 9.7% to 11.4% for the α1,3 variant. The same holds true for non-fucosylated glycan species, where the relative amounts of the upper (α1,6)- vs. lower (α1,3)-arm galactosylated isomer were 2.4% and 0.9%, respectively, for HILIC(2-AB), and between 1.4% to 2.4% and 0.6% to 1.3%, respectively, for all other methods. The relative amount of doubly galactosylated, fucosylated species G2F was determined to be 9.6% for the HILIC(2-AB). This is nearly the same as found for CE-LIF(APTS-HR1) (Table 2). With HILIC(IAB), a slightly higher result was found (10.4%), and for the other methods slightly lower results (ranging from 8.2% to 9.5%) were observed. The sum of non-fucosylated species (G0+G1+G2), an important parameter for
antibody effector function, averaged 8.4% for HILIC(2-AB), and nearly the same for HILIC(IAB) at 8.6%. All other methods featured lower amounts of non-fucosylated species, namely DSA-FACE(APTS) 7.1%, CCGE(ANTS) 6.5%, CE-LIF(APTS-HR2) 7.8% and HPAEC-PAD 7.1%. CE-LIF(APTS-HR1) could not detect G2, thus the lowest relative amount of non-fucosylated species (6.2%) was obtained with this method. However, since G2 is quantitated significantly below 1%, the influence of G2 on the afucosylation level is low.

The sum of monoantennary structures (structures lacking an N-acetylglucosamine G0F-N, G1F-N and G0-N) was found to average 0.9% for HILIC(2-AB). It must be noted, however, that G1F-N could not be fully separated. No monoantennary structures were resolved with CCGE(ANTS), and with CE-LIF(APTS-HR1) and DSA-FACE(APTS), only G0-N could be separated and quantified (relative amount 0.4% and 0.3%). The other methods showed similar relative amounts of monoantennary structures with 0.8% for CE-LIF(APTS-HR2), 0.9% for HILIC(IAB) and 1.3% for HPAEC-PAD.

For the M5 species, an average relative abundance of 1.5% was detected with HILIC(2-AB). Similar values were found for the other methods (ranging from 1.5% to 2.2%).

The sum of sialylated structures (G1FS, G2S1F and G2S2F) was found to be 1.0% with HILIC(2-AB). CE-LIF(APTS-HR1), CE-LIF(APTS-HR2), DSA-FACE(APTS) and HPAEC-PAD showed relative amounts ranging from 0.5% to 1.1%. HILIC(IAB) and CCGE(ANTS) exhibited the highest values, 1.7% to 1.9%, respectively.

**Analysis time and throughput**
The information concerning analysis time and throughput is shown in Table 3. All methods employed were based on the analysis of glycans. The first step is always the release of the glycans, which is one of the most time-consuming steps. For the reference method, four hours sample preparation time for 6 samples is needed, out of which about 1.5 hours is hands-on time. In principle, there is not much difference between the methods. The fluorescence-based methods need labeling and clean-up steps. HPAEC-PAD was faster (1.5 hours sample preparation) because no labeling step is required. The sample preparation for all methods can be automated; however, the DSA-FACE(APTS) method is the only method that is really a high-throughput method since 96 samples can be analyzed in parallel.

**Skills and investments needed**
As indicated in Table 3, no special skills were needed for any of the methods, but the analyst should be properly trained in the sample preparation and the instruments. For the fluorescence-based methods, a HPLC or CE system is required. Additionally, labeling kits must be purchased from time to time, which could contribute significantly to the cost.

**Required sample amount and purity**
An overview is shown in Table 3. For the Reference Method and HILIC(IAB), interference from contaminants was not observed, but, since we used formulated bulk material as the sample, which is intrinsically very pure, the presence of contaminants from other samples that interfere with labeling could not be excluded. The same holds true for the CE-based methods. Contaminant interference for
APTS and ANTS was not observed. For HPAEC-PAD, in principle, there could be problems with agents other than oligosaccharides that would also separate with a weak anion exchange column.

HILIC(2-AB) started with 200 µg of sample, and HILIC(IAB) with 50 µg. However, since the fluorescence detection for 2-AB is very sensitive, it should be possible to detect glycans in the femtomol quantities.\textsuperscript{67}

The amount of sample was 300 µg for CE-LIF(APTS-HR1), 50 µg for CE-LIF(APTS-HR2) and 5 µg for DSA-FACE(APTS). These amounts were not optimized for the lowest amounts of mAb1. Adamczyk \textit{et al.}\textsuperscript{33} estimated the detection limit of APTS-labeled glycans by CE-LIF to be 0.4 nM.

CCGE(ANTS) required an initial amount of 50 µg. However, as ANTS should have a comparable sensitivity to APTS, here also a detection limit of about 0.4 nM could be estimated. HPAEC-PAD started with 400 µg of sample; the detection limit for glycans, however, should be far below this amount.
<table>
<thead>
<tr>
<th>Method</th>
<th>Analysis time and throughput</th>
<th>Skills and investment needed</th>
<th>Required purity and sample amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>HILIC(2-AB) Reference Method</td>
<td>4 h sample preparation (1.5 h hands-on time); 10 h for 6 samples including separation; sample preparation can be automated</td>
<td>No special skills for the analyst needed; equipment: HPLC or UPLC with fluorescence detection; 2-AB labeling kits needed</td>
<td>No interference from contaminants observed; 200 µg mAb1</td>
</tr>
<tr>
<td>HILIC(IAB)</td>
<td>1.5 to 2 h sample preparation (1 – 1.5 h hands-on time); 8 h for 6 samples including separation; sample preparation expandable to 96 well plates; can be easily automated</td>
<td>No special skills for the analyst needed but must be properly trained in using the sample preparation system; equipment: HPLC or UPLC with fluorescence detection; 2-AB labeling kits needed</td>
<td>No interference from contaminants observed, 50 µg mAb1</td>
</tr>
<tr>
<td>CE-LIF(APTS-HR1)</td>
<td>24 h for 6 samples (5 h hands-on time); sample preparation and data evaluation can be automated but problems with robustness of the system</td>
<td>No special skills for the analyst needed but must be properly trained in using the sample preparation system; equipment: any CE-system with fluorescence detection; APTS labeling kits needed</td>
<td>No interference from contaminants observed; 300 µg mAb1</td>
</tr>
<tr>
<td>DSA-FACE(APTS)</td>
<td>For 96 samples 2-3 h hands-on time; optimally suited for high-throughput; 96 samples can be analyzed in parallel</td>
<td>No special skills for the analyst needed; DNA Analyzer with capillary technology needed, APTS labeling kits needed</td>
<td>No interference from contaminants observed; 5 µg mAb1</td>
</tr>
<tr>
<td>CE-LIF(APTS-HR2)</td>
<td>3 h for 6 samples (1-1.5 h hands-on time); electrophoretic analysis 35 min; total preparation time for 6 samples is 3.5 h; expandable to 96-well plates and easily automatable</td>
<td>No special skills for the analyst needed but must be properly trained in using the sample preparation system; equipment: any CE-system with fluorescence detection; APTS labeling kits needed</td>
<td>No interference from contaminants observed; 50 µg mAb1</td>
</tr>
<tr>
<td>CCGE(ANTS)</td>
<td>3 h for 6 samples (1.5 – 2 h hands-on time; total preparation and analysis time for 6 samples 3.5 h; expandable to 96 well plates and easily automatable</td>
<td>The analyst must be properly trained in using the sample preparation system; developmental CE is needed; special labware is needed; ANTS labeling kits needed</td>
<td>No interference from contaminants observed; 50 µg mAb1</td>
</tr>
<tr>
<td>HPAEC-PAD</td>
<td>Sample purification takes 30 min; separation time 1.5 h for one sample (hands-on time for six samples 1 h); no need for high-throughput for sample preparation</td>
<td>No special special skills for the analyst are needed; Investment: a HPLC system that is suited for the high-pH buffers and equipped with a pulsed amperometric detector</td>
<td>No interferences observed, but oligosaccharide and non-oligosaccharide contaminants might be of concern; 400 µg mAb1</td>
</tr>
</tbody>
</table>
Discussion

Taken together the results obtained with all separation methods without mass spectrometric detection – with regard to the detection and quantitation of glycoforms – were very similar. With the exception of HPAEC-PAD, where the detection is based on amperometry, the other methods are based on fluorescence detection. The robust and comparable quantification of results is most likely due to the fact that only one fluorophore is added to the reducing end of the glycans. The detection with 2-AB-labeling is known to be very sensitive (femtomol quantities), but there may be a bias caused by partial glycan degradation during the labeling process, where the loss of the sialic acid could be of particular concern. We found no clear evidence for sialic acid degradation during labeling. A minor loss of sialic acid may occur with the standard 2-AB-labeling protocol (2 h at 65°C under acidic conditions) because only 1.0% sialic acid-containing glycans was found in comparison to 1.8% with InstantAB, where labeling takes place instantly at room temperature and neutral pH. The fluorophore used for three CE-based methods was APTS, where sialic-acid degradation may also occur during labeling. Additionally, electrokinetic injection was applied, and this might favour glycans with high mobility, i.e., sialylated species, since in CE separation the charged species migrate first. Such an effect was not seen in the study as the quantitative results of the main species for CE-based methods were similar to non-CE-based methods.

In general, it is a challenge to robustly quantitate sialylated species with CE-based methods, as they may co-migrate with other species. However, our showed in the case of the novel CCGE(ANTS) method very similar results for the relative quantitation of sialylated species to those obtained with HILIC(IAB) method. This particularly good performance of CCGE(ANTS) for the analysis of sialylated species might be due to the label used (ANTS instead of APTS which is used for the other CE methods) or to the chosen separation conditions. Notably, with regard to the main species, very similar results were obtained for all fluorescence detection-based methods, so there is no evidence for a labeling bias of different glycostructures.

HPAEC-PAD showed slight differences in galactosylation levels compared to the other methodsExisting scientific literature suggests that the response for different glycostructures varies in HPAEC-PAD, but that the effect is low. Increasing glycan size and sialylation is believed to cause reduction in PAD response. This might account for the small differences for G0F and the sialylated species.

Coming to the drawbacks of the methods: the following remarks can be made: With HILIC(2-AB) G1F-N could not be separated and in general the method is relatively time consuming. As stated before there could be an underestimation in sialic acid quantitation due to a bias in the labelling process. Concerning the CE-based methods as mentioned before also the loss of sialic acid may be of concern and some peaks are co-migrating. as to the HPAEC method there is the concern that one has to apply response factors for the different glycostructures, as discussed before. To circumvent these issues it is always a good choice to use two different methods.

99
In summary all seven methods showed excellent performance for accuracy, precision and separation, and are well suited for the purpose of analyzing Fc-glycosylation of IgG1. The relative quantitation for the individual glycan species were comparable. In principle, all methods could be used as release methods, and validating them should be no problem. In our hands, the Reference Method, HILIC(2-AB), is optimally suited for release. The method found to be best suited for high throughput was DSA-FACE(APTS), where 96 samples can be analyzed in parallel. If all glycan structures of a mAb must be quantified, the use of two methods in parallel is advised. All methods in the study exhibited excellent standard deviations and low day-to-day variability. The situation is more diverse with sialylated species, as methods with rapid-reductive-amination labeling detected higher amounts. All methods described and tested here could in principle also be applied for Fc-fusion proteins, bispecific antibodies and glycoengineered antibodies as well as other glycoproteins. However when sites-specific information is essential mass spectrometry based methods might be more useful. In the second part of our report, the mass spectrometry-based methods will be presented and an overall comparison between all methods will be given.

Materials and Methods

MAb1 was produced in a CHO cell line, and purified by the Downstream Processing Group at Roche Diagnostics GmbH.

HILIC(2-AB) (The Reference Method)

MAb1 (200 µg, 350 µl) was buffer exchanged with the aid of Nanosep’ centrifugal devices (Pall, USA) to ammonium formate buffer (10 mM, pH 8.6). N-glycosidic-bound oligosaccharides were released by incubating 48-µl samples with 2 µl PNGase F (500,000 U/ml, New England Biolabs) at 45°C for 1 h. Released glycans were labeled with 2-AB at 65°C for 2 h (Glyko® Signal 2-AB Labeling Kit, ProZyme). Excess 2-AB was removed using HyperSep-96 Diol cartridges (Thermo) with a vacuum station. Labeled glycans were washed with 96% acetonitrile, eluted from the cartridges and analyzed by HILIC-UHPLC using a Waters BEH Glycan Separation Technology column (2.1 x 150 mm, 1.7µm) on a Dionex RSRC Ultimate 3000RS or a Waters ACQUITY UPLC® system. A 45-min acetonitrile gradient was applied and fluorescence signals were detected at 420 nm (excitation at 330 nm). Peaks were integrated automatically according to pre-defined parameters with the software Chromeleon© and relative glycan compositions were calculated.

HILIC(IAB)

MAb1 (50 µg) was prepared using GlykoPrep® Rapid N-Glycan Sample Preparation (GS96-RX - GlykoPrep Digestion Module); the sample was mixed with Denaturation Reagent and incubated at room temperature for 5 min. The denatured protein mixture was applied to the RX Cartridges, where the protein is immobilized and deglycosylated with PNGase F for 30 min on a heatblock set to 50°C. The released N-glycans were eluted and immediately labelled at room temperature (without incubation) with GlykoPrep InstantAB™ (GS96-LB - GlykoPrep InstantAB Labeling Module). Labeled N-glycans were then cleaned up using CU Cartridges to remove excess labeling reagents.
InstantAB-labeled glycans were then analyzed by UPLC-FLR using a Waters BEH-Glycan Separations Technology column (1.7 μm, 150 x 2.1 mm, part number 180064742) with an increasing ammonium formate gradient (mobile phase A: Acetonitrile; mobile phase B: 100 mM Ammonium formate at pH 4.4) over 60 min. An injection volume of 1 μl aqueous and a column temperature of 35°C were used; glycans were detected at a wavelength of 330 nm with an excitation wavelength of 278 nm. Peaks were integrated using Empower® software (Waters Corp.), and relative glycan compositions were calculated.

**CE-LIF(APTS-HR1)**

MAb1 was diluted to approximately 10 mg/ml (30 µl) with product formulation buffer using a Microcon-30 concentrator (Amicon). PNGase F diluted in reaction buffer (50 mM sodium succinate pH 5.5) was added, and the sample was incubated for approximately 15 h at 37°C. The deglycosylated protein was heated and precipitated by centrifugation. The supernatant was dried and reconstituted in an excess solution of 15 µl of acidic APTS (ProZyme) (5 mg in 0.5 ml of 15% v/v glacial acetic acid) and 5 µl of 1 M sodium cyanoborohydride in tetrahydrofuran (Aldrich). This solution was heated at 55°C for 2 h. The solution was diluted with water to a final volume of 250 µl.

CE-LIF experiments were performed using a Beckman Coulter PA800 plus Pharmaceutical Analysis System with LIF detection (ex: 488 nm; and em: 520 nm). Separation was performed with Beckman eCAP neutral capillaries (60 cm total length; 50 cm effective length; 50 µm ID; 360 µm OD; Beckman Coulter); running buffer was a 50/50 mixture of carbohydrate separation buffer and DNA gel buffer (Beckman Coulter); an applied voltage of -30 kV. Capillaries were kept at 20°C and flushed with running buffer prior to each analysis. No additional conditioning was used. Injection was performed hydrodynamically at 0.5 psi for 10 s.

Peaks were integrated automatically according to pre-defined parameters with the software 32-Karat© (% corrected peak area) and relative glycan compositions were calculated.

**DSA-FACE(APTS)**

MAb1 (5 µg) was transferred to AcroPrepTM Advance 96-Well Filter Plates 30K Omega from Pall and water was added to give a final volume of 300 µl. The plates were centrifuged three times after addition of 300 µl of water for five times with 1500xg.

Samples were reconstituted in 50 µl of water containing 1 µl of PNGase F (250 U of enzyme were dissolved in 250 µl water). Filter plates were sealed and the samples were directly incubated on the filter at 37°C overnight. The released glycans were separated from IgG via the filter plates by centrifugation for 5 min at 1500xg into 96-well receiver plates (ProZyme). Samples were dried by vacuum centrifugation.

Labeling was performed with the GlykoPrep® Rapid-Reductive-Amination APTS Labeling Module for 96-well plates (ProZyme, GS96-APTS), consisting of reductant solution, APTS solution and APTS catalyst solution. For 96 samples, typically 104 µl of reductant, 260 µl of APTS catalyst and 104 µl of APTS solution were mixed. Dried glycans where reconstituted in 4.5 µl of the prepared
APTS-labeling master mix, the plates were sealed and labeling performed with light excluded for 4 h at 50°C. Clean up after labeling was performed with GlykoPrep Clean Up (CU) Cartridges (GS96-C2, ProZyme). Then 20 ml of 5x APTS sample loading buffer (ProZyme) was filled up to 100 ml with acetonitrile. Samples were then diluted in 200 µl APTS sample loading buffer with thorough mixing and subsequently loaded onto the CU Cartridges using 3 min centrifugal force at 300xg followed for 1 min at 1000xg. Then CU Cartridges were washed with 2 times 200 µl of APTS sample loading buffer by centrifugation for 3 min at 300xg to remove excess dye and labeling side products. Finally samples were eluted with 2 times 50 µl of water by centrifugation for 3 min at 1000xg.

For 96 samples 1250 µl Hi-Di Formamide (Applied Biosystems product code 4311320) was mixed vigorously with 3.5 µl of Basepair Size Standard (Applied Biosystems 500 Rox Size Standard product code 401734). Cleaned up samples were diluted 1:20 with water. Prior to analysis 2 µl of diluted samples were then mixed with 10 µl HI-DI Formamide Basepair Size Standard mixture.

Analyses were performed with a 48-capillary array (50-cm length; filled with Pop-7™ Polymer (Applied Biosystems). Injection was performed with an injection voltage of 3 kV for 15 sec; separations were performed at 15 kV over a run time of 1800 s. Data analysis was performed by an in-house-developed Matlab application. The software normalized the migration time on the internal base pair standard by a regression function of 2nd polynomial order. The area of the assigned peaks was determined and the relative area of the glycans were calculated.

**CE-LIF(APTS-HR2)**

MAb1 (50 µg) was prepared using GlykoPrep® Rapid N-Glycan Sample Preparation (GS96-RX - GlykoPrep Digestion Module); the sample was mixed with Denaturation Reagent (ProZyme) and then incubated at room temperature for 5 min. The denatured protein mixture is applied to the RX Cartridges, where the protein is immobilized and deglycosylated with PNGase F for 30 min on a heat block set to 50°C. The released N-glycans are eluted and immediately treated with Finishing Reagent and incubated for 10 minutes on a 50°C heat block to convert the released glycans to the reduced, aldehyde form; N-glycans are then dried in a centrifugal evaporator, followed by labeling with GlykoPrep Rapid-Reductive-Amination™ APTS (GS96-APTS - Reductive-Amination APTS Labeling Module). Labeled N-glycans were purified using CU Cartridges to remove excess labeling reagents. APTS-labeled glycans were analyzed by CE-LIF with a Beckman Coulter PA800 plus Pharmaceutical Analysis System using a Beckman-Coulter N-CHO capillary (50 µm inner diameter, 60 cm total length – 50 cm effective, part number 477601) with a separation voltage of 20 kV (reversed polarity) over 35 min. The buffer used was a 1:1 mixture of N-Linked Carbohydrate Separation Gel Buffer and eCAP™ ds DNA 1000 Gel. An injection protocol of 2 psi for 10 seconds and a capillary temperature of 20°C were used. Glycans were detected at a wavelength of 520 nm with an excitation wavelength of 488 nm. Peaks were integrated using Empower® software (Waters Corp) and relative glycan compositions were calculated.
CCGE(ANTS)

MAb1 (50 μg) was prepared using GlykoPrep® Rapid N-Glycan Preparation (GS96-RX - GlykoPrep Digestion Module); the antibody is mixed with Denaturation Reagent and then incubated at room temperature for 5 min. The denatured protein mixture is applied to the RX Cartridges, where the protein is immobilized and deglycosylated with PNGase F for 30 min on a heatblock set to 50°C. The released N-glycans are eluted, immediately treated with Finishing Reagent and incubated for 10 minutes on a 50°C heatblock to convert the released glycans to the reduced, aldehyde form. Reduced glycans are then dried in a centrifugal evaporator. Dried glycans are labeled with a developmental labeling kit utilizing (ANTS) as the fluorescent tag. Labeled N-glycans are cleaned up using CU Cartridges to remove excess labeling reagents. ANTS-labeled glycans are analyzed by CE-FLR using a developmental cartridge-based capillary gel electrophoresis system (capillary: 75 μm inner diameter, 15.5 cm total length – 11.5 cm effective) with a separation voltage of 6 kV (reversed polarity) over 4 min. The buffer used contained a gel matrix. An injection protocol of 3 kV for 10 s was used and the capillary was operated at ambient temperatures; glycans were detected at a wavelength of 530 nm with an excitation wavelength of 420 nm. Peaks were integrated using Empower® software (Waters Corp) and relative glycan compositions were calculated.

HPAEC-PAD

MAb1 (400 μg) was transferred to NAP5©-columns (GE-Healthcare 17-0853-02) and buffered with 10 mM sodium phosphate pH 7.2 and concentrated to a final volume of 50 μL. Subsequently the sample was incubated with 2 Units of N-Glycosidase F (Roche 1 365 193) for 16 h at 37°C. Vivaspin concentrators (0.5 ml; 10 kD; Sartorius, (Göttingen, Germany) were used to separate the glycans from residual protein. The remaining solution was washed two times with 30 μl of water and diluted with water to give a final volume of 140 μl. HPAEC-PAD experiments were performed using a BioLC equipped with a CarboPAc PA200 column (ThermoFisher Scientific, Bremen, Germany). Mobile phase A was 50 mM NaOH and mobile phase B was 50 mM NaOH, 200 mM sodium acetate. The glycans were eluted using a gradient to give 3% Eluent B after 25 min, 20% Eluent B after 55 min and 70% Eluent B after 90 min. A flow rate of 0.5 mL/min was used; the injection volume was 10 μl. Detection was by means of pulsed amperometric detection Peaks were integrated automatically according to pre-defined parameters with the software Chromeleon©, and relative glycan compositions were calculated.

Acknowledgments

M. Wuhrer was supported by the European Union’s Seventh Framework Program (FP7-Health-F5–2011) under grant agreement n°278535 (HighGlycan).

Abbreviations

mAb, monoclonal antibody;  Fc, fragment crystallizable;  IgG, immunoglobulin G; HILIC-UPLC, Hydrophilic interaction liquid chromatography-ultra high performance liquid chromatography; 2-AB, 2-aminobenzamide; Fab, fragment, antigen-binding; CE-LIF, capillary electrophoresis- laser induced
fluorescence; HPLC, high performance liquid chromatography; MALDI-MS, matrix-assisted laser desorption/ionization-mass spectrometry; ESI-MS, electrospray ionization-mass spectrometry; HPAEC-PAD, high-performance anion exchange chromatography with pulsed amperometric detection; APTS, 8-aminopyrene-1,3,6-trisulfonic acid; DSA-FACE, DNA-sequencer-aided fluorophore-assisted carbohydrate electrophoresis; ANTS, 8-aminonaphthalene-1,3,6-trisulfonate; CCGE, cartridge-based capillary gel electrophoresis; HR, high resolution; IAB, InstantAB labeling; CHO, Chinese hamster ovary.
References