Methods for the glycosylation analysis of therapeutic antibodies

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Chapter 8:

General Discussion

The aim of this thesis was to develop high-throughput methods for analyzing the Fc glycosylation of therapeutic antibodies. Moreover in this thesis various methods for IgG glycosylation analysis available are compared and evaluated in their usefulness for different purposes such as release, comparability or process development. Finally, the impact of Fc glycosylation of therapeutic antibodies on patient safety, immunogenicity, bioactivity and pharmacodynamics/pharmacokinetics was evaluated on the basis of data available in the literature.

Development of high-throughput methods for glycosylation analysis of therapeutic antibodies

First, two high-throughput methods for the analysis of Fc glycosylation of therapeutic antibodies were developed (chapters 3 and 4). One method relies on the mass spectrometric detection of glycopeptides and the uses capillary CE separation on a DNA analyzer for detecting released glycans after labeling with a fluorophore. Both methods are now used routinely in the laboratories at Pharma Biotech Development in Penzberg, Germany in the context of the extensive characterization of large molecules. More than 10000 samples are analyzed a year in this unit.

Capillary electrophoresis with a DNA Analyzer

Benefits of the DNA Analyzer

The high-throughput glycosylation analysis of therapeutic immunoglobulin G by capillary gel electrophoresis is advantageous because no mass spectrometer is needed. Instead, the method relies on a DNA analyzer which makes this technique rather easily accessible to different laboratories due to the wide availability and comparatively modest costs of this equipment. In the pharma environment not all laboratories have mass spectrometric equipment and it is especially difficult to transfer a mass spectrometric method from development laboratories to quality control laboratories. Furthermore glycan analysis on the DNA analyzer is uniquely advantageous due to the multiplexing capacity: Up to 96 samples can be analyzed at once. This can be very helpful in cases when the results of the analysis are needed quickly in order to support process development. With the internal standard (DNA base pair size mixture) the migration times can be normalized and the resulting normalized migration positions are adequately reproducible between samples and runs. As the detection with laser induced fluorescence is very sensitive, the samples can simply be diluted after labeling and analyzed without any post-labeling purification.

Our work was performed using 96 wells ELISA plates enabling the analysis of 48 samples at once (see Chapter 4). With the development of new equipment by different vendors it is

expected that capillary heads with 384 capillaries will become available which may further increase sample throughput. In general we expect that the glycan characterization with a DNA analyzer could also be run in non-specialized laboratories or in an at-line analytics setting near the fermentation process. For this purpose there is still a need to reduce the hands-on time and to automate the data evaluation.

Drawbacks of the DNA Analyzer

The time consuming step in this procedure is the labeling with APTS. But with the availability of instant labeling APTS this step could be accelerated considerably ([1-3] and *http://www.prozyme.com*). The method is not able to separate all glycans that are present in IgG (see Chapter 4). However it is assumed that new polymers (enabling separation in the capillaries) will be available in the near future.

When we started with our method development only second-hand DNA Analyzers were available. This could be disadvantageous as there is the risk that within approximately 10 years from now it may not be possible to get service from the vendor. But as the market for this kind of equipment is attractive, it is expected that other vendors will develop more miniaturized and easy-to-use instruments [2]. What is still missing is the direct structural elucidation of the glycan species as to date the DNA analyzer cannot be hyphenated to mass spectrometry. We addressed this issue by including a HILIC-MS separation as first dimension, peak fractionation and subsequently analysis of peaks with the DNA analyzer in comparison with commercially available glycan standards (see Chapter 4). Bunz *et al.* used another strategy and reported a capillary electrophoresis system (but not on a DNA analyzer) directly coupled to ESI-MS by using an acidic background electrolyte (BGE) and could show in their study unequivocal peak assignment for all unknown glycans in a glycan mixture of medium complexity released from a recombinantly expressed fusion protein [4].

Application of the DNA Analyzer method for other purposes

The DNA Analyzer method could in principle also be used for the glycosylation analysis of other samples. As the method is based on released glycans, there must be a separation step at the glycopeptide level before the glycan release in order to maintain site specificity. For Fab glycosylation analysis of therapeutic antibodies using a DNA analyzer there has to be prior separation of the Fc and the Fab part in order to allow unambiguous site assignment. The same holds true if IgGs with glycosylated receptor or cytokine domains have to be analyzed.

If glycoproteins like erythropoietin (EPO; with multiple N-glycosylation sites with tri- and tetraantennary structures) have to be analyzed there are several challenges such as purification from cell broth and large complexity of the sample. To reduce complexity the samples may be desialylated with neuraminidase. Doing so the information concerning site specific sialylation gets lost but the relative amounts of triantennary structures, tetraantennary structures and

tetraantennary structures with repeats can be evaluated. Together with the absolute quantification of released sialic acid (for example by releasing the sialic acid with neuraminidase, labeling the sialic acids with a fluorescence label (1,2-diamino-4,5-methylenedioxybenzene) and measuring the fluorescence) the glycosylation of EPO samples can be sufficiently characterized [5;6]. As exemplified with EPO, biopharmaceuticals with highly complex N-glycosylation will therefore most probably require additional methodology, next to N-glycan analysis with the DNA analyzer, to describe their glycosylation in sufficient detail for most of the applications.

Conclusion

The developed N-glycan analysis method using a DNA analyzer is fast, robust and cost-efficient, and has great potential for the use in all laboratories involved in glycomics which are not experienced in mass spectrometry-based glycoanalytics. In fact this is supported by several studies of similar methods that can be applied for clinical research [7-10].

In future the method can be made even more robust and we have plans to establish it as an open access analytical method where people from other departments may come to our lab and measure their samples themselves. We expect also that easy-to-use kits for APTS labeling and kits for labeling with other labels that are more sensitive in laser induced fluorescence will become available in the coming years which will improve the accessibility of this method even more.

Glycopeptide method for high-throughput

The second high-throughput method for glycan analysis that was developed is based on direct measurement of the intact glycopeptides. After tryptic digestion and purification with Sepharose® beads (HILIC) the glycopeptides are directly infused into an ESI-MS mass spectrometer.

Benefits of the glycopeptide method

With the glycopeptide analysis method there is no need for labeling, as it is a mass spectrometry based approach. Moreover, the method provides site-specific glycosylation information. An advantage of the method is that no separation dimension is required such as CE and HPLC. In case the sample preparation is performed in a 96-well plate, it can be fully automated (see chapter 3). If the samples are antibodies with a Fab glycosylation, the Fc glycosylation and the Fab glycosylation can be evaluated separately [11]. From the peptide moiety it can be deduced on which site the glycan was bound to the protein.

The sample preparation method has been originally developed for use with MALDI-MS [12]. If ESI-MS is used for detection, automation appears to be easier to achieve as the purified samples can automatically be injected into the mass spectrometer by means of a robotic system. However

data evaluation is somewhat more challenging as multiple charge states are produced with ESI-MS.

Drawbacks of the glycopeptide method

A disadvantage of the HILIC purification is that the concentration of acetonitrile with sample loading and washing often appears to be critical. Small variations in the HILIC purification (for example acetonitrile content) come with the risk of the selective partial loss of some glycan species leading to inaccuracy of the relative quantification. To address this, reference material has to be used as a standard in each series functioning as a system suitability test. With mass spectrometric methods there is always the risk that the glycans are fragmented (see chapter 2). As the developed method was also included in the method comparison (chapter 6) this phenomenon is discussed in the following section.

Application of the glycopeptide method for other purposes

In principle different glycoproteins could be analyzed with this approach provided that they can be digested with trypsin or another protease that produces peptides with only a limited number of amino acids.

Other methods for high-throughput glycosylation analysis

There are also other methods available for high-throughput glycosylation analysis of therapeutic antibodies. The Caliper System is a chip based microfluidic system and allows fast, automated, electrophoretic separations of released glycans. However a dedicated CE system is needed for these analyses[13].

With the mAb-Glycochip system the deglycosylation of the antibody, the glycan separation and direct transmission to the mass spectrometer occurs on a chip. The samples are analyzed consecutively with run times of 5 minutes. However there is the restriction that an Agilent mass spectrometer has to be used [14].

Conclusion

With regard to technological advancement of biopharmaceutical glycosylation analysis, innovations may arise from the (clinical) glycomics field where it is mandatory to have high-throughput methods. New technologies are emerging that can be adapted to the methods needed in the pharmaceutical industry [15-18]. Especially methods used for analysis of blood samples might be easy to adapt, as blood is, like fermentation broths, a very complex matrix.

Methods for the analysis of therapeutic immunoglobulin G Fc-glycosylation profiles

In this thesis an extensive study was performed comparing methods for the analysis of therapeutic IgG Fc-glycosylation. The first part is dedicated to the separation based methods and the second part to the mass-spectrometric based methods (chapters 5 and 6). It is a considerable challenge to compare analytical methods in an objective manner. Performance differences between laboratories and operators may bias the results considerably. Also there has to be a comparable data evaluation. We tried to avoid biases by using methods that were well-established in the different labs and by ensuring that only experienced analysts did the experiments that were part of the study. In total four laboratories were involved. In order to take into account potential inter- and intra-day six replicates of each method were measured on two different days and mean values and standard deviations calculated accordingly. We are confident that the results obtained with the different methods gave reliable results that are really representative of the involved methods, and not of performance differences between labs or operators.

Some of the methods that we included in the mass spectrometry study are also separation based. But as glycans show negligible UV absorption the detection can only be done with mass spectrometers (LC-MS of tryptic digest, PGC-MS). Therefore they were included in the mass spectrometry part of the study (see chapter 6).

Reference method

As the reference method we used HILIC-UPLC after AB-labeling, which in our hands is robust and gives reliable results. It is the method of choice if glycosylation analysis is needed for release or for quality related purposes like comparing material used in different clinical phases (Comparability). It has the advantage that the characterization of the peaks can be done by comparing the retention times of the individual glycans with a database (by comparing so called Glucose-Units) [19]. Additionally it can easily be transferred to quality control units and has proved to be easy to validate. (Non published results).

Separation-based methods

Some separation based methods have the advantage that they are able to separate isobaric structures. For IgG glycosylation analysis for example the alpha 1,3 and the alpha 1,6 mannose bound G1F structure cannot be distinguished with mass spectrometry based methods but is separated with all separation based methods tested.

The separation based methods (see chapter 5) can be divided in three groups: 1. HPLC separation after labeling with a fluorophore 2. Capillary electrophoresis after labeling with a fluorophore and 3. HPLC without prior modification of the glycans. The latter comprises only HPAEC-PAD (see chapter 5). It is a method that is still used in pharmaceutical laboratories but it is increasingly being replaced with UPLC after AB labeling.

Some methods could not separate all peaks (see chapter 5).

One challenge is to assign and characterize the minor peaks. If the separation based methods can be hyphenated to a mass spectrometer MS/MS experiments can be performed. However for the time being it is not clear whether health authorities require these minor peaks to be characterized and the relevance of peaks below 1% is open to be discussed. The glycan G2F has the same molecular mass as G1F with a galactose that is alpha 1,3 linked to the galactose. As alpha 1,3 galactose may be immunogenic (see chapter 2) the two species have to be distinguished and quantified separately. The distinction cannot be made with a mass spectrometry based method. We saw that especially PGC-MS is well suited to separate these two glycan species (unpublished results). For the other separation based methods this still needs to be demonstrated, for example using commercially available standards.

A benefit of the separation based methods compared to the mass spectrometry based methods is that only simple software is needed to perform the data processing (quantitation).The quantitative results for the main peaks for all separation based methods were very comparable.

Mass spectrometry based methods

In principle - independent of the quantitative analysis - mass spectrometric methods are more useful if site specific information and in-depth characterization of the glycosylation pattern is essential. With fragmentation experiments by applying MS/MS detailed characterization can be achieved. However it is still a challenge to establish mass spectrometers in quality control laboratories. The transfer of mass spectrometric methods to other labs is also not easy to accomplish and non availability of trained technicians might cause problems.

If the Fc glycosylation of antibodies that are glycoengineered by overexpression of GnTIII [20] has to be analyzed (see chapter 2) there are many more isobaric structures present (the introduced bisecting N-acetylglucosamine makes the characterization with only mass spectrometric methods ambiguous). The strategy in this case would be to separate the released and AB-labeled glycans with reversed phase (RP) HPLC instead of HILIC HPLC, as RP chromatography seems to be well suited to separate fucosylated from non-fucosylated glycans and to hyphenate the HPLC to an electrospray mass spectrometer. All isomers can be separated and all peaks assigned by doing MS/MS experiments [21]. In general using a non-mass spectrometry based method for routine analysis once MS/MS has been applied to achieve the assignment of peaks might be a useful strategy.

One has to be careful as with mass spectrometry based methods as system-induced fragmentation might occur. This is especially the case if glycans with a small degree of sialylation have to be analyzed (see chapter 6). Fucose rearrangements may also occur, complicating structural assignment on the basis of MS/MS data [22].

Methods with and without prior separation

We included methods in the study with (LCMS of tryptic digests) and without prior separation (direct infusion of purified glycopeptides). The advantage of the direct infusion method is that it is a high-throughput method and a large number of samples can be analyzed automatically. On the other hand, because there is no chromatographic separation involved and absolute purification of glycopeptides is difficult to achieve then ion suppression might occur if too many non-glycosylated peptides are co-purified. The LCMS based methods on the other hand need more hands-on time and are more laborious. As retention time in the separation dimension is quite long the LC-MS methods that are routinely used are not suited for high-throughput and have to be adapted. Therefore UPLC methods with shorter run times should be used to analyze more than 100 samples a day which seems not to be feasible with conventional HPLC equipment. However, despite the significantly longer run time, the benefits of a LCMS run is the extensive amount of information that can be obtained from it. For example, not only information on the glycosylation but also, other post translational modifications like methionine and tryptophan oxidation, deamidation, succinimidylation, C- and N-terminal species can also be evaluated. Unexpected impurities can be detected as well. If MS/MS is employed even the sequence of amino acids can be deduced [23].

MALDI-MS versus ESI-MS

Two major technologies for mass spectrometers differentiated by the mechanisms of ionization were included in the study, namely matrix assisted laser desorption ionization mass spectrometry (MALDI-MS) and electrospray ionization mass spectrometry (ESI-MS). ESI-MS was not used to measure released glycans but to measure the glycosylation in the form of the glycopeptides (bottom up) and intact mass after reduction of disulfide bridges (top down) or after digestion with for example IdeS (middle down; see chapter 6). MALDI-MS is not well suited to measure higher molecular masses with a satisfying resolution. But it can be well used to measure glycans and glycopeptides (see chapter 6). However if low amounts of sialic acid are present on the outer arm of the oligosaccharide this information is ambiguous in the MALDI process. This can be precluded by stabilizing the sialic acids before analysis. To date this method is available for glycans but it would be helpful to use this method also for glycopeptides and intact glycoproteins [24].

Bottom-up, middle-down and bottom-down

The problem with the top-down and middle-down strategies is that if more post translational modifications are in the part that bears the glycosylation, interpretation of data is hampered and quantitation of glycan species is quite difficult. For example if there is a glycation of lysine present, it has the same mass increment as an additional hexose and for structures differing in one hexose quantification is therefore compromised. However as the sample preparation for the

top-down and middle-down methods are fast they might be the methods of choice if there are a large number of samples to be analyzed and if not only certain aspects of the glycosylation (profile) is needed. As such, we expect that these methods will be adopted in many labs but they will not replace the established released glycans analytical methods.A strategy could be to deglycosylate the therapeutic antibody with PNGase F and then to apply two methodologies: One to analyze the released glycans (with the methods for released glycans mentioned in chapters 5 and 6) and the other to measure the intact deglycosylated protein (ESI-MS) to gain information about the integrity of the antibody, as well as about glycation, cysteinylation and other post translational modifications.

The different mass spectrometers used had an influence on the results obtained. The most prominent difference was the loss of an N-acetyl-glucosamine and special care has to be taken when tuning the instrument that this loss is minimized (see chapter 6).

Challenges and future trends

Requirements of glycoanalytical methods for biotherapeutics

The requirements of glycoanalysis methods for therapeutic antibodies in the biotech industry depend on the intended use. If the methods are to be used for release they require validation. This means they have to be accurate and robust so that someone who is trained according to the standard operating procedure for the method produces always the same results for a reference sample. For the other purposes, no extensive validation is required and it is only necessary to demonstrate conclusively that the method is suitable for its intended use

The methods should be robust. The hands-on time should be minimized and full or partial automation is mandatory. For method selection the sample amount available also has to be taken into account. If only limited amounts of sample are available miniaturization could be helpful in the future. Also the choice of the protease for digestion (if glycopeptides are analyzed) is also an important consideration.. For speeding up the whole sample preparation procedure fast digests with proteases like trypsin or PNGase F for released glycans could be helpful.

For characterizing the glycans other, more specific glycosidases could also be used (for example Endoglycosidase H for high-mannose glycans). Additionally exoglycosidases like sialidase, galactosidase and mannosidase could be used to characterize the glycans.

Application of the methods studied

One of the scopes of our study was to evaluate which is the most appropriate method for the different purposes. For release we prefer the 2-AB HILIC method for standard IgG and 2-AB with RP-HPLC for glycoengineered antibodies where it is important to register the afucosylation level. In our quality control laboratory capillary electrophoresis subsequent to APTS labeling is also used as a release method.

For clone selection, methods are required that can process a large number of samples with often low amounts of protein. In our hands these methods are the two high-throughput methods described in chapter 3 and 4. The DNA analyzer has the greatest potential for at-line analytics in the fermentation unit. However it is always advisable to have two methods available so that if there are problems with one method, the other method can be used for troubleshooting.

Challenges

If small amounts of N-glycolylneuraminic acid (NGNA) are present on the glycans, their detection is also important as they may be immunogenic (see chapter 2). This is also the case when Sp2/0 or NSO cells have been used to produce the therapeutic antibody. Although we have not demonstrated it in our study, it should be possible with all included methods to analyze NGNA.

With some of the methods it is not possible to detect all expected glycostructures. For example if there is a "non-consensus" N-glycosylation site present in a therapeutic antibody it cannot be released with PNGase F. To circumvent this the "consensus glycans" can be released with PNGase F and the species with the non-consensus glycosylation site (intact molecule or glycopeptide) can then be concentrated with Lectin columns and subsequently analyzed [25]. We did not include any methods for O-glycosylation determination in our study as therapeutic antibodies normally do not exhibit any O-glycosylation. However in recent years some unexpected O-glycans like O-Fucose were found in IgG and methods hence need to be developed to allow their detection [26].

Future trends

For all methods described herein, the quantification was relative. It could be advantageous and more informative if absolute quantification would be used. It would be very valuable to compare the degree of sialylation obtained in this study with the results from absolute quantification methods. This could be performed by quantitative measurement of the DMB labelled sialic acids after release of the sialic acids with sialidase [6]. The use of isotopically labeled internal standards can also be applied to achieve absolute quantification in mass spectrometric based methods [27]. More and more "high end" mass spectrometry equipment like the Orbitrap are used for glycosylation analysis. It is envisioned that mass spectrometers with a better sensitivity and mass accuracy may be available in the future. It might be possible to separate isobaric structures by means of ion mobility [28;29]. Glycoproteins could be used as biomarkers and the task of glycoanalysis will be to distinguish between the glycosylation fingerprint of for example a malign and a benign state. In the next years antibodies against specific glycosylated proteins could be developed and integrated in diagnostic tests.

Conclusion of method comparison

The quantitative evaluation of the data for all methodsevaluated here yielded very similar results which was contrary to expectations, as prior studies reported considerable differences between results for the same sample coming from different laboratories [30;31]. The National Institute of Standards and Technology (NIST) has called for participation in a huge international interlaboratory study concerning analyzing IgG glycosylation.

See: *http://chemdata.nist.gov/dokuwiki/doku.php?id=chemdata:glyco_call*.

Impact of Fc glycans and in vitro glycoengineering

The third part of the study described in this thesis provides an evaluated summary of our knowledge on the impact of Fc glycosylation on patient safety, drug immunogenicity, bioactivity and pharmacodynamics/pharmacokinetics. Additionally an investigation of the the influence of galactosylation and sialylation of an IgG on receptor binding and ADCC after *in vitro* glycoengineering is provided.

Information from published literature

In general it is difficult to draw conclusions on structure function relationships from the available literature due to controversial findings. For example in the case of the impact of the galactosylation of Fc glycans on Fc receptor binding and ADCC of the antibody, there are reports that demonstrate an impact and others the contrary (see chapter 2). Nevertheless, some structure-function relationships have unambiguously been demonstrated: There are no doubts in the literature that afucosylation influences FcγIII receptor binding and consequently ADCC (antibody-dependent cellular cytotoxicity).

Future Structure-function relationship investigations with the help of in vitro glycoengineering

In vitro glycoengineering is a convenient tool to produce glycoproteins with a defined glycosylation pattern. However for the time being a certain limitation is the high cost and/or lack of availability of the necessary glycosyltranferases and the activated sugars. Maybe the enzyme and the activated sugars will become more easily available in the near future, making *in vitro* glycoengineering more viable. Certain glycoforms are still not accessible for functional evaluation, even not with in vitro glycoengineering: While it would be interesting to also investigate the influence of monogalactosylated structures (G1F) in particular G1F 1,3 and G1F 1,6 it is not possible to separate the two species in the native state (at the level of the therapeutic antibody). We tried to produce G1F with *in vitro* glycoengineering, but the procedure required several steps with long incubation times. So other post translational modifications occurred that compromised the results in the assay for receptor binding and ADCC (unpublished results).

Also the impact of alpha2,3 and alpha2,6 bound N-acetyl-neuraminic acid has to be evaluated. In our study we found no significant impact of sialylation on ADCC (see chapter 7), however there

have been contrary reports from other groups [32]. We used beta-galactosamide alpha-2,6-sialyltransferase to produce alpha2,6 bound sialic acid. Maybe there is also an influence if alpha2,3 bound sialic acid is produced (with beta-galactoside alpha-2,3-sialyltransferase). Moreover, the number of sialic acids per glycan chain, as well as the sialylation of alpha 1,3 versus the alpha 1,6 arm in monosialylated bi-antennary glycans may play a role. Furthermore the impact of NGNA has to be shown in future studies.Coming to pharmacokinetics and pharmacodynamics it would be helpful to produce therapeutic antibodies with the Man5 structure (by using kifunensine in the fermentation process and trimming the high mannose structures with mannosidases to Man5 [33]) and with the G0F-N structure to do human PK/PD studies to evaluate the impact of these glycostructures.

Conclusion

We think that in vitro glycoengineering will have a great potential for the use in CQA assessment (see chapter 2 and 7).Furthermore we are increasingly able to produce antibodies with desired properties. So therapeutic antibodies with a defined glycosylation pattern might move from experimental use to therapeutic applications in the clinic with a big market potential.

Overall conclusion

In general it is difficult to predict the future role of glycoanalysis and in vitro glycoengineering. More and more studies show the importance of glycosylation for therapeutic glycoproteins and in living organisms. As such, it is logical consequence that the demand will increase for more laboratories to provide glycoanalysis methods. This could be in the form of more on-site labs or glycoanalysis service laboratories. In any case, mass spectrometry will certainly continue to play an important role in glycoanalysis as it is the technology to characterize glycans in detail. However due to the superior nature of these technologies it is likely they will remain restricted to specialized laboratories in the near future. The broad dissemination of mass spectrometric glycoanalytical methods is not foreseen for the coming years, due to the considerable complexity of these analytical approaches.

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