Methods for the glycosylation analysis of therapeutic antibodies

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Chapter 1: Introduction

Therapeutic antibodies

"It is possible to hybridize antibody producing cells from different origins. Such cells can be grown in vitro in massive cultures to provide specific antibody. Such cultures could be valuable for medical and industrial use"[1]. These are the final and predictive words of the publication of the Nobel Prize laureates Milstein and Köhler in 1975. Today therapeutic monoclonal antibodies produced in the biopharmaceutical industry are of key importance in the treatment of various diseases and are among the top-selling medicines world-wide [2].

Immunoglobulins (antibodies) are an important part of the humoral immune response in humans. They can be divided into five different isotypes, namely IgA, IgD, IgE, IgG and IgM. They can be found in different parts of the body and fulfill different functions. Immunoglobulins are built by a combination of heavy and light chains: there are five isotypes of heavy chains (γ, μ, α, ε, σ) and two isotypes of light chains (λ and ϰ) [3].

IgGs are the most abundant antibodies in blood and lymph. Four IgG subclasses are distinguished: IgG1, IgG2, IgG3 and IgG4. For therapeutic use exclusively IgG1, IgG2 and IgG4 are used. Each IgG is comprised of two heavy chains and two light chains which are linked by disulfide bonds forming a Y-shaped molecular structure. A major difference between the IgG subclasses are the number of disulphide bonds in the hinge region, namely two for IgG1 and IgG4, four for IgG2 and eleven for IgG3 [4].

In order to create diversity for antigen binding the amino acid sequence at the top of each of the Y-arms is varied. Consistently, this is called the variable region or Fab (Fragment antigen binding). The lower part of the light chain and the lower hinge region of the heavy chain is called Fc (fragment crystallizable) [5].

IgG normally has its glycosylation site at asparagine 297 in the Fc part. This thesis deals with the development and comparison of methods for IgG1 Fc glycosylation analysis (see Chapter 2).

The history concerning production of therapeutic antibodies can be credited to some famous researchers (all of them were Nobel laureates). Emil von Behring found an inducible and specific immunity through immunization with pathogens. Individual animals could be protected by transferring serum from immunized animals. He called the active substance Antitoxin [von Behring E. and Kitasato.S, The mechanism of diphtheria immunity and tetanus immunity in animals. 1890, *Molecular Immunology,* Volume28, Issue12 (1991) 1317, 1319-20].

Paul Ehrlich postulated in 1900 the so-called side-chain theory: living cells have side-chains that can link with a particular toxin. After contact with the antigen the cell grows additional side-chains and they break off. These were the magic bullets or antibodies [P.Ehrlich, The Theory and Practice of Chemotherapy *Folia Serologica,* 7 (1911) 697]. In 1962 Rodney Porter and Gerard Edelman deduced the chemical structures of antibodies (Quaternary structure with two heavy chains and two light chains). Then in 1965 L. Craig and N. Hilschmann discovered the variable regions. Niels Jerne (Nobel Prize 1984) brought up the concept of clonal selection.

As introduced, Georges Köhler and Cesar Milstein (1975) invented the hybridoma technology and with this the production of monoclonal antibodies. Myeloma cells and B cells are fused and hence antibodies with a certain specificity for an antigen could be produced [1].

As these mouse antibodies had non-human amino acid sequences and non-human glycosylation (see Chapter 2) they showed immunogenic properties when applied in human therapy. To address this, chimeric antibodies were developed where the sequence of the Fc was human and the Fab was of mice origin. Subsequently only the hypervariable part (CDR, complement determining region) was of mouse origin and the other part of the therapeutic antibody was "humanized". Nowadays fully human therapeutic antibodies can be produced by phage display [6], primary B-cell PCR (poly chain reaction)[7] or transgenic animals like mice or rabbits [8]. As host cells for producing the antibodies most often CHO cells are used in the biotech industry but also NS0 and SP2/0 cells are in use [9].

Of the different isotypes, IgGs are the class that have found their way most frequently into therapeutic use. Today monoclonal antibodies are used for many disease areas including oncology (non-small cell lung cancer, melanoma, neuroblastoma, gastric cancer, colorectal cancer, breast cancer and many more), autoimmune diseases (rheumatoid and psoriatic arthritis, ankylosing spondylitis, psoriasis, Crohn's disease, multiple sclerosis and many more) and infectious diseases (anthrax infection) [2;10]. Hundreds of therapeutic antibodies are in development and more than fifty monoclonal antibodies are approved or in review in the European Union or the United States [11] (see Table 1). As can be seen in Table 1 three to six new therapeutic monoclonal antibodies are approved every year and the rate of new approval appears to be steadily increasing.

Table 1. Therapeutic antibodies approved or in review in the European Union or the United States.

International non-proprietary name	Tradename	Target; Format	Indication first approved or reviewed	First EU approval year	First US approval year
Alirocumab	(Pending)	PCSK9; Human IgG1	High cholesterol	In review	In review
Mepolizumab	(Pending)	IL-5; Humanized	Severe eosinophilic	In review	In review
Necitumumab	(Pending)	EGFR; Human IgG1	Non-small cell lung	In review	In review
Nivolumab	Opdivo	PD1; Human IgG4	Melanoma	In review	2014
Dinutuximab	(Pending)	GD2; Chimeric IgG1	Neuroblastoma	In review	NA
Secukinumab	Cosentyx**	IL-17a; Human IgG1	Psoriasis	EC decision pending	In review
Evolocumab	(Pending)	PCSK9; Human IgG2	High cholesterol	In review	In review
Blinatumomab	Blincyto	CD19, CD3; Murine	Acute lymphoblastic	In review	2014
Pembrolizumab	Keytruda	PD1; Humanized	Melanoma	In review	2014
Ramucirumab	Cyramza	VEGFR2; Human IgG1	Gastric cancer	EC decision pending	2014
Vedolizumab	Entyvio	α4β7 integrin; humanized	Ulcerative colitis, Crohn	2014	2014
Siltuximab	Sylvant	IL-6; Chimeric IgG1	Castleman disease	2014	2014
Obinutuzumab	Gazyva	CD20; Humanized	Chronic lymphocytic	2014	2013
Ado-trastuzumab emtansine	Kadcyla	HER2; humanized IgG1;	Breast cancer	2013	2013
Raxibacumab	(Pending)	B. anthracis PA; Human	Anthrax infection	NA	2012
Pertuzumab	Perjeta	HER2; humanized	Breast Cancer	2013	2012
Brentuximab vedotin	Adcetris	CD30; Chimeric IgG1;	Hodgkin lymphoma, systemic anaplastic	2012	2011
Belimumab	Benlysta	BLyS; Human IgG1	Systemic lupus	2011	2011
Ipilimumab	Yervoy	CTLA-4; Human	Metastatic melanoma	2011	2011
Denosumab	Prolia	RANK-L; Human	Bone Loss	2010	2010
Tocilizumab	Actemra	IL6R; Humanized	Rheumatoid arthritis	2009	2010
Ofatumumab	Arzerra	CD20; Human IgG1	Chronic lymphocytic	2010	2009
Canakinumab	Ilaris	IL1b; Human IgG1	Muckle-Wells syndrome	2009	2009
Golimumab	Simponi	TNF; Human IgG1	Rheumatoid and psoriatic arthritis,	2009	2009
Ustekinumab	Stelara	IL12/23; Human	Psoriasis	2009	2009
Certolizumab pegol	Cimizia	TNF; Humanized Fab,	Crohn disease	2009	2008
Catumaxomab	Removab	EPCAM/CD3;Rat /mouse bispecific	Malignant ascites	2009	NA

International non-proprietary name	Tradename	Target; Format	Indication first approved or reviewed	First EU approval year	First US approval year
Eculizumab	Soliris	C5; Humanized	Paroxysmal nocturnal	2007	2007
Ranibizumab	Lucentis	VEGF; Humanized IgG1	Macular degeneration	2007	2006
Panitumumab	Vectibix	EGFR; Human IgG2	Colorectal cancer	2007	2006
Natalizumab	Tysabri	a4 integrin; Humanized	Multiple sclerosis	2006	2004
Bevacizumab	Avastin	VEGF; Humanized	Colorectal cancer	2005	2004
Cetuximab	Erbitux	EGFR; Chimeric	Colorectal cancer	2004	2004
Efalizumab	Raptiva	CD11a; Humanized	Psoriasis	2004#	2003#
Omalizumab	Xolair	IgE; Humanized	Asthma	2005	2003
Tositumomab-I131	Bexxar	CD20; Murine IgG2a	Non-Hodgkin lymphoma	NA	2003#
Ibritumomab tiuxetan	Zevalin	CD20; Murine IgG1	Non-Hodgkin lymphoma	2004	2002
Adalimumab	Humira	TNF; Human IgG1	Rheumatoid arthritis	2003	2002
Alemtuzumab	Campath-Lemtrada	CD52; Humanized IgG1	Chronic myeloid leukemia#;	2001#; 2013	2001#; 2014
Gemtuzumab ozogamicin	Mylotarg	CD33; Humanized	Acute myeloid leukemia	NA	2000#
Trastuzumab	Herceptin	HER2; Humanized	Breast cancer	2000	1998
Infliximab	Remicade	TNF; Chimeric IgG1	Crohn‘s disease	1999	1998
Palivizumab	Synagis	RSV; Humanized	Prevention of respiratory	1999	1998
Basiliximab	Simulect	IL2R; Chimeric IgG1	Prevention of kidney transplant rejection	1998	1998
Daclizumab	Zenapax	IL2R; Humanized	Prevention of kidney transplant rejection	1999#	1997#
Rituximab	MabThera, Rituxan	CD20; Chimeric	Non-Hodgkin lymphoma	1998	1997
Abciximab	Reopro	GPIIb/IIIa; Chimeric IgG1	Prevention of blood clots in angioplasty	1995*	1994
Muromonab-CD3	Orthoclone Okt3	CD3; Murine IgG2a	Reversal of kidney transplant rejection	1986*	1986#

Legend: ** Approved in Japan in December 2014; *, Country-specific approval; # Withdrawn; NA, not approved or in review (Source: Janice M. Reichert, PhD, Reichert Biotechnology Consulting LLC; table updated January 17, 2015)

To improve antibody function many different strategies have been applied. The properties of the Fc were altered by modifying the glycosylation and thereby improving the antibody-dependent cell-mediated cytotoxicity (ADCC) through enhancement of the binding to Fc receptors (ADCC, see Chapter 3). An alternative strategy involves specific engineering of the Fc to increase binding to the FcRn (neonatal Fc receptor; prevents IgG degradation). Antibody-drug conjugates were developed with additional functionality through conjugation to drugs like toxins or cytokines. Another novel and versatile form of biopharmaceuticals are bispecific antibodies which bind to two different antigens [10;12-14]. In 2009 the first bispecific antibody catumaxoman (Removab; Fresenius Biotech/TRIon Pharma) was approved by the European Medicines Agency (EMA) for the treatment of malignant ascites. It combines the binding to epithelial cell adhesion molecule (EPCAM) on cancer cells and CD3 on effector cells [15]. Many other bispecific antibodies are in development [13]. Although it is so far not possible to produce identical copies of antibodies because of their structural complexity (one of the main issues being glycosylation) there are now regulatory pathways to bring biosimilar antibodies to market [13;16].

Since the prevalence of diseases like cancer and infectious diseases is globally increasing, also the biopharmaceuticals market (today estimated to be >199 billion US Dollar (Source: Research and Markets, Biopharmaceuticals – A Global Market Overview, M2 Press WIRE 2013) is expected to increase considerably. The development and commercialization of therapeutic antibodies, currently the biggest part of biotherapeutics, will therefore be of utmost importance for the pharmaceutical industry.

Glycosylation analysis of therapeutic antibodies

Glycans of therapeutics produced in mammalian cell lines are built from a limited number of monosaccharides. The most abundant are galactose (Gal), mannose (Man), fucose (Fuc), N-acetylglucosamine (GlcNAc), N-acetylgalactosamine (GalNAc) and N-acetylneuraminic acid (NeuAc, NANA). Oligosaccharides are formed by linking together different monosaccharides.In some cases also a glycosylation on the Fab part of the therapeutic antibody may occur. However this was not in the focus of the work presented here. The most common examples of glycosylation types are protein-linked N-glycans and O-glycans, next to glycosphingolipids and proteoglycans. The studies in this PhD thesis focuses on protein N-glycosylation. The N-glycosylation site is characterized by the sequon Asn-X-Ser/Thr (where X is not proline but can be any other amino acid). The biosynthesis of N-glycans takes place in the endoplamatic reticulum (ER) where a block of sugars (Glc3Man9GlcNAc2) is transferred to the nascent protein at the amino group of Asn (therefore N-glycosidic linkage). The glycan chain is then processed in the ER and the golgi apparatus. Three different types of N-glycans are generated: high-mannose type glycans, hybrid glycans and complex glycans (see Figure 1). IgG glycosylation in the Fc part yields only biantennary structures.

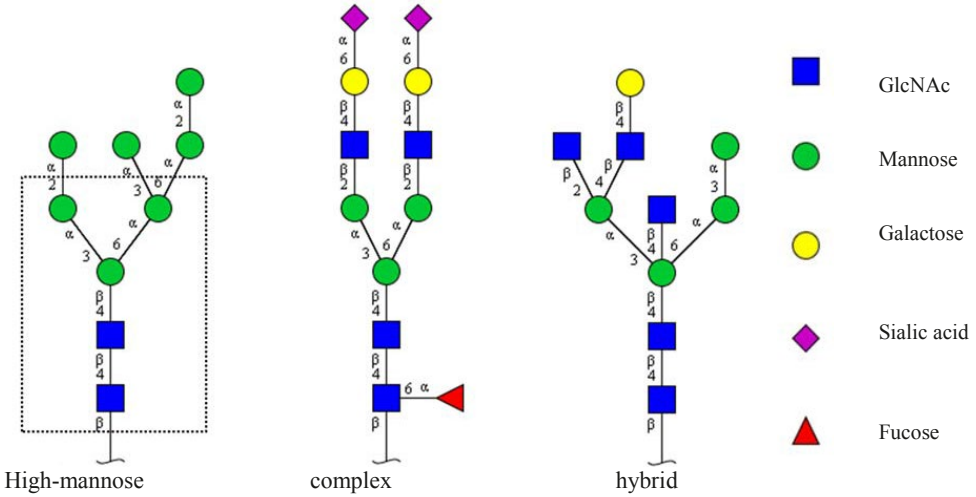

Figure 1. High-mannose-type, complex and hybrid N-glycosylated glycan structures.

Different oligosaccharides can be found at the same glycosylation site. This complexity arises from the diversity of the structure of the monosaccharides, the sequence of the monosaccharides and the linkages between them. Accordingly the theoretical number of oligosaccharides is very high but because of the biosynthetic pathway (see Figure 2) the actual number is significantly smaller. The final glycosylation is a

reflection of the biological environment, for example, the expression system used for production.

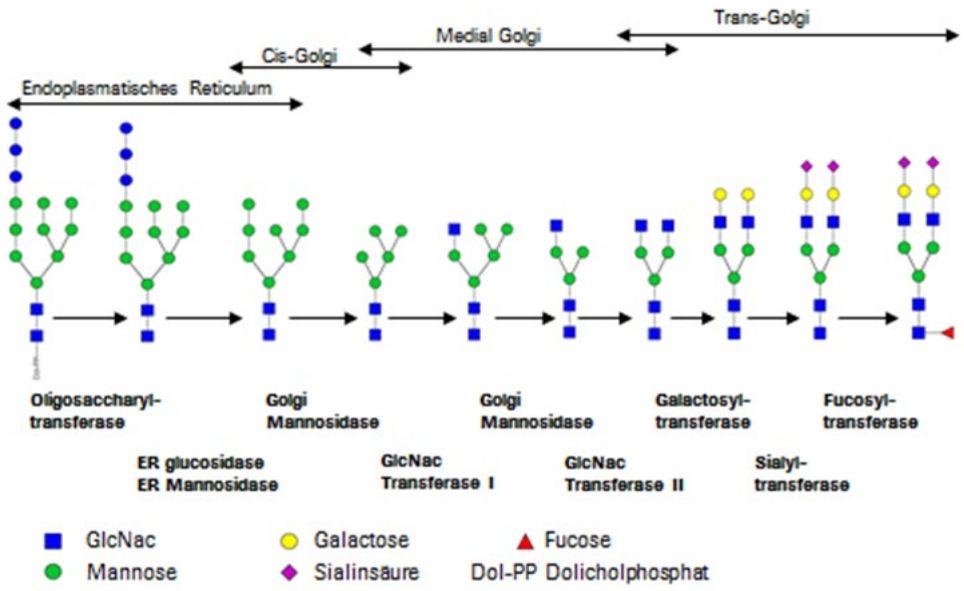

Figure 2. Biosynthesis of N-glycans.

The glycosylation profile of biotherapeutics has to be consistent and to a large extent human-like to prevent adverse events [17]. Therefore monitoring of the IgG glycosylation is of great importance in cell line development, clone selection, process characterization and process validation studies, as well as for comparability purposes and release. Regulatory agencies released guidelines concerning glycoanalysis. For example the EMEA (European Medicines Agency) issued a guideline thatdefines quality requirements for monoclonal antibodies. In chapter 4.3 (CHARACTERISATION OF MONOCLONAL ANTIBODIES) of the "GUIDELINE ON DEVELOPMENT, PRODUCTION, CHARACTERISATION AND SPECIFICATIONS FOR MONOCLONAL ANTIBODIES AND RELATED PRODUCTS (EMEA/CHMP/BWP/157653/2007)" the following requirements are specified concerning glycoanalysis: "The carbohydrate content (neutral sugars, amino sugars and sialic acids) should be determined. In addition the structure of the carbohydrate chains, the oligosaccharide pattern (antennary profile), the glycosylation site(s) and occupancy should be analyzed. Typically, monoclonal antibodies have one N-glycosylation site on each heavy chain located in the Fc-region [...] Glycan structures should be characterized, and particular attention should be paid to their degree of mannosylation, galactosylation, fucosylation and sialylation. The distribution of the main glycan structures present (often G0, G1 and G2) should be determined."

There are different reasons why glycoanalysis is a challenge:

1. Complexity and diversity; glycans have an inherent diversity with respect to the number of isomers (monosaccharides), antennarity and spatial distribution

2. Glycans are polar, thermically instabile and do not have a chromophore, compromising both separation and detection

3. Several structures are isobaric and hence cannot be distinguished with MS based techniques

4. Glycopeptides and glycans exhibit low ionization efficiencies as compared to non-modified (less polar) peptides

To overcome these challenges many different analytical technologies are employed in the biotech industry. Methods for glycoanalysis of biotherapeutics especially for Fc glycans were reviewed in several manuscripts [18-20]. In principle three strategies for glycoanalysis can be employed:

1. Analysis of the intact molecule and deduction of the overall glycan composition

2. Enzymatic or chemical release of the glycans and measurement of the glycans

3. Proteolytic cleavage and measurement of the glycopeptide

Some are separation based, while others are mass spectrometry based. The various strategies are schematically summarized in Figure 3.

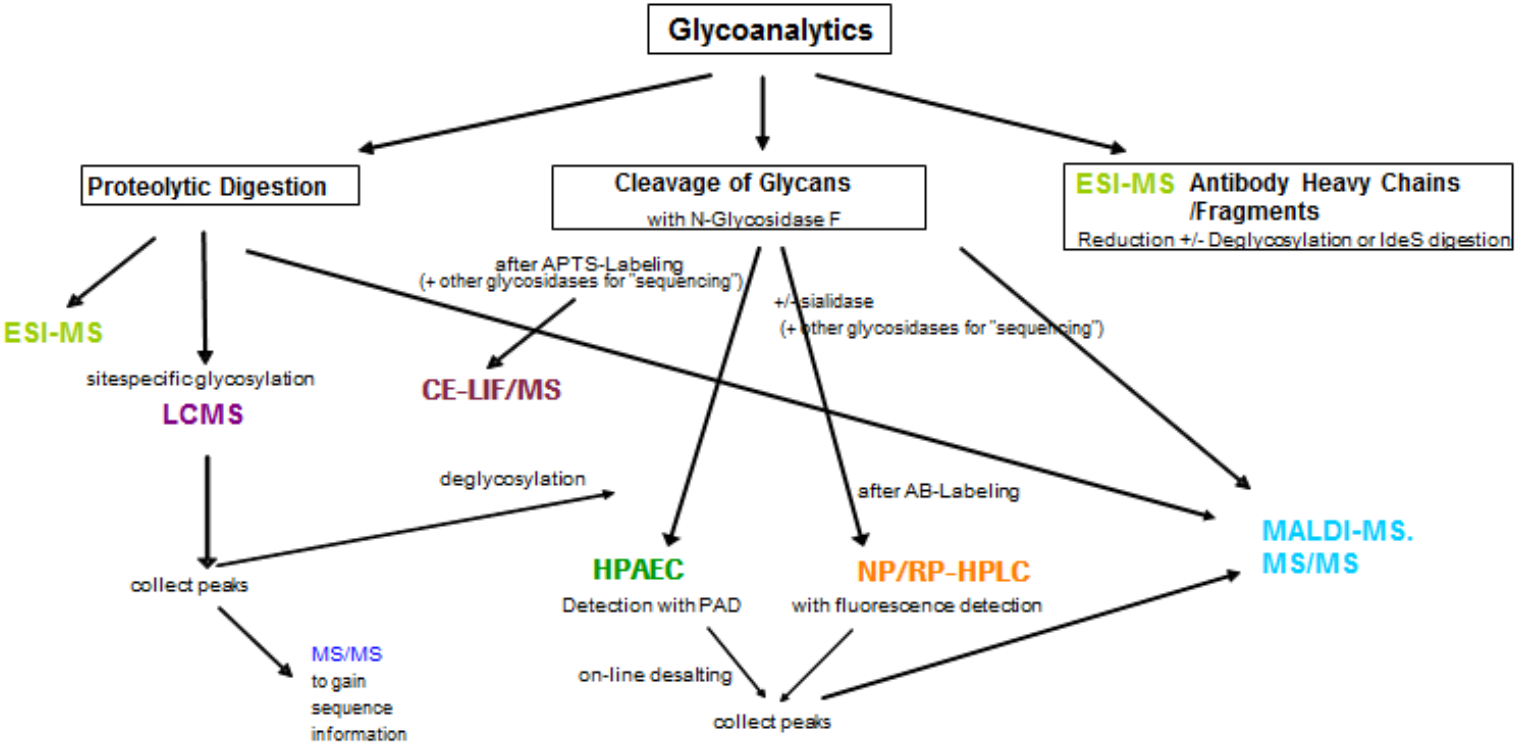

Figure 3. General strategies for glycoanalysis.

If a protein is glycosylated or not can be evaluated by staining with periodic acid-Schiff reaction (after SDS-PAGE separation) or by a Lectin blot [21;22].

The analysis of the intact IgG molecule can be performed with electrospray ionization mass spectrometry (ESI-MS), either on the intact molecule after reduction of the disulphide bonds or after digestion with Endoproteinase LysC, Pepsin or IdeS. As mentioned before the overall glycan composition can be deduced from the molecular mass with and without glycans. However since there are also other post translational modifications these methods have some drawbacks [23-25]. The N-linked glycans can be released from the therapeutic glycoprotein with the enzyme N-glycosidase F (PNGase F) or with other endoglycosidases like Endo H, Endo F or Endo S. PNGase F cleaves the linkage between the core N-acetyl-glucosamine and the asparagine residue of the glycoprotein, the asparagine is thereby transformed to aspartic acid.

As glycans do not possess a chromophore they need to be made amenable to detectionwith chromatography and electrophoresis separation based methods through derivatization. By introducing fluorophors the chromatographic and electrophoretic properties of the glycans for separation are enhanced and the sensitivity for detection is improved. Also for mass spectrometry based methods due to the decreased polarity the ionization efficiency and therefore signal intensities are enhanced. Commonly used fluorescent dyes for glycans are, for example, 2-aminobenzamide (2-AB) for chromatography based methods [26] and APTS (8-aminopyrene-1,3,6-trisulfonic acid) or ANTS (8-aminonaphtalene-1,3,6 trisulfonate for electrophoresis of glycans [27]. One method without prior derivatization that is not mass spectrometry based is available. The glycans are separated with high pH anion exchange HPLC and detection is with pulsed amperometric detection (HPAEC-PAD) [28]. The most promising non-mass spectrometric methods for the analysis of released glycans are hydrophilic interaction chromatography subsequent to AB-labeling and capillary electrophoresis with laser-induced fluorescence (CE-LIF) following APTS labeling [19].

Glycans can also be measured without prior derivatization with matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) and electrospray ionization mass spectrometry (ESI-MS) [18]. As ionization efficiencies for sialylated glycans are generally low, and detection is furthermore compromised by the lability of sialic acid glycosidic linkages, glycans can be derivatized for example by amidation or esterification of the sialic acids. Another approach for glycoanalysis is measurement of the cleavedglycopeptides. With this approach, the therapeutic antibody is proteolytically cleaved with proteases such as trypsin, LysC or pronase and the released glycopeptides are measured [29]. With mass spectrometric analysis of glycopeptides an in-depth analysis of IgG glycosylation in a site-specific manner is possible and also the Fab glycosylation can be measured.

If further characterization of the glycans with respect to structure and linkage is required, additional methods for the extended characterization have to be employed. The conformation of a glycan can be elucidated by NMR (nuclear magnetic resonance). As glycans can exist in multiple conformations in solution, acquisition of a set of orthogonal NMR observables is preferable [30]. Linkage analysis can be done with mass spectrometric methods with or without prior permethylation [31-34].

It is also possible to quantitate the sialylation of a given glycan by hydrolyzing the sialic acid or by digesting the glycoprotein/glycan with a sialidase. The sialic acid content can then be measured without prior modification with HPAEC-PAD or stable isotope dilution using high-performance liquid chromatography-tandem mass spectrometry [35]. After modification with a fluorescent label for example o-phenylenediamine (OPD) and separation with HPLC the sialic acids can be measured with a fluorescence detector [36]. In the biotech industry for process development, medium development, and clone selection there is a need for high-throughput glycoanalysis. Therefore this thesis focuses on the development of high-throughput techniques for glycoanalysis and the comparison of methods for glycoanalysis.

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Scope

Therapeutic monoclonal antibodies are very successful and promising biotherapeutics for the treatment of e.g. autoimmune diseases, in ophthalmology and cancer. They are glycoproteins and are N- glycosylated on their Fc part (see Introduction). The detailed characterization of these glycans on therapeutic antibodies is extremely important in the biotech industry since changes of glycosylation can have significant impact on the bioactivity, pharmacokinetic and pharmacodynamics behavior and safety for patient. In the pharmaceutical industry glycan analysis is needed for clone selection, comparability, process development, release of products and other purposes. A lot of different methods for glycan analysis are available. They are based on the analysis of the intact molecule, glycopeptides or glycans. The methods can also be divided in separation based methods and mass spectrometry based methods. It is advantageous to use orthogonal methods to evaluate all features of glycosylation. Therefore a comparison of the different methods could be helpful. For process development and other purposes high-throughput methods are needed that allow the measurement of many samples directly from the fermentation broth to support upstream and downstream processing. The scope of this thesis was to develop miniaturized high-throughput sample preparation and analysis strategies for the more detailed characterization of IgG Fc glycosylation by mass spectrometry and separation based methods. Furthermore this work aims at comparing available methods for glyco analysis of therapeutic antibodies and to evaluate the impact glycosylation patterns can have.

In **Chapter 2** a review focuses on the role of Fc glycans as critical quality attributes (CQAs). Fc glycan information from published literature as well as internal data are summarized and evaluated for impact on patient safety, immunogenicity, bioactivity and pharmacodynamics/pharmacokinetics.

Chapter 3 describes the development of a high-throughput work flow for IgG Fc-glycosylation analysis of IgGs from fermentation broths. Glycopeptides are purified by hydrophilic interaction solid-phase extraction and analyzed by electrospray mass spectrometry.

In **Chapter 4** the development of a high-throughput method for the analysis of therapeutic immunoglobulin G by capillary gel electrophoresis using a DNA analyzer is described. Moreover the practical value of the developed method was demonstrated by analyzing the antibody glycosylation profiles from fermentation broths after small scale protein A purification.

In **Chapter 5** and **Chapter 6** a comparison of methods for the analysis of therapeutic immunoglobulin G Fc-glycosylation profiles are described. Special emphasis was placed on the detection of sialic acid-containing glycans. It focuses in the first part on the non-mass spectrometric methods that are separation-based and on the mass spectrometric methods in the second part. Moreover and overall conclusion for both parts of the method comparison is given in the second part.

Chapter 7 describes the in vitro glycoengineering of IgG1 and its effect on Fc receptor binding and ADCC (antibody-dependent cell-mediated cytotoxicity) activity. In this study the influence of IgG1 Fc galactosylation and sialylation on its effector function were investigated using samples produced by *in vitro* glycoengineering. Finally, a general discussion is given in **Chapter 8.**