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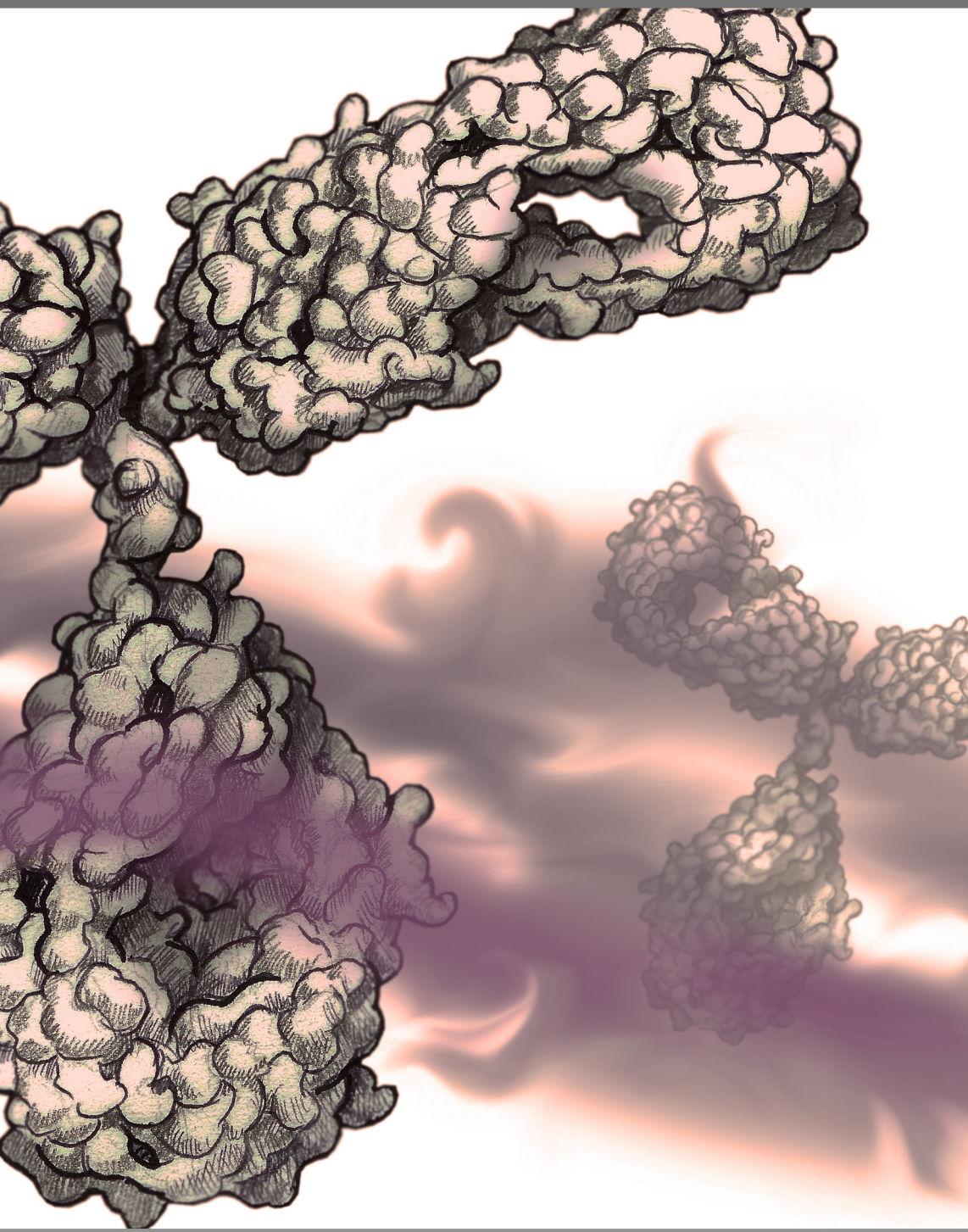
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GLYCOSYLATION ANALYSIS OF IMMUNOGLOBULIN G

method development and clinical applications



**Glycosylation analysis of immunoglobulin G:
method development and clinical applications**

Maurice Henricus Johannes Selman

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IgG glycosylation analysis

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Introduction

Immunoglobulins (Igs) represent an important part of the human immune system and can adapt to many different antigenic challenges. Structural variety of Igs is achieved by the expression of hypervariable regions which create the antigen binding site and define the antigen specificity and affinity. Moreover, various classes (i.e. IgM, IgD, IgG, IgA, IgE) and subclasses (e.g. IgG1-4) of immunoglobulins are present in humans, differing in temporal expression patterns as well as in function. IgG represents the most abundant antibody class in the human circulation, being present at a concentration of approximately 10 mg/ml.^{1,2} Two major application fields for IgG glycosylation analysis have evolved, first IgGs in the human circulation as part of the humoral immune response, and second biotechnologically produced monoclonal antibodies for therapeutic purposes.

IgG consists of two heavy chains and two light chains. Parts of the heavy chains, together with the light chains, form two fragment antigen binding (Fab) moieties, whilst the remainders of the two heavy chains form the fragment crystallizable (Fc) moiety. Next to the enormous variety in the sequence of the antigen-binding region of plasma IgG, human IgGs vary in the N-glycosylation at the highly conserved N-glycosylation site asparagine 297 in the C_H2 domain of each heavy polypeptide chain of the Fc part. As a general pattern, most IgG Fc N-glycans are biantennary complex-type structures, which often carry a core-fucose (Fuc) and vary in the number of antenna galactose (gal) residues (Figure 1A).^{3,4} Some of the glycans contain a bisecting N-acetylglucosamine (GlcNAc) and a small portion may carry a sialic acid (SA) residue attached to an antenna galactose. Notably, the largest variation has been reported regarding galactosylation: a varying portion of the IgG Fc N-glycoforms is truncated. The biantennary, core-fucosylated IgG Fc N-glycoforms with no, one, or two galactoses are referred to as G0F, G1F, and G2F, respectively. The portion of IgG Fc N-glycoforms that carry no galactose is particularly large for rheumatoid arthritis (RA) patients, as has been described by Parekh *et al.* in 1985.³ This is demonstrated in Figure 1, which shows IgG Fc N-glycosylation profiles of a healthy control (B) and an RA patient (C).

The second field of IgG glycosylation analysis has experienced a considerable growth in recent years. With the advent of therapeutic monoclonal IgGs, the engineering and analysis of IgG Fc N-glycosylation, Fab glycosylation as well as O-glycosylation, together with other posttranslational modifications, has become an important analytical challenge in the pharmaceutical industry. The number of licensed monoclonal antibodies (mAbs) is steadily increasing. There are over 30 approved therapeutic mAbs worldwide and hundreds in clinical trials or in development.⁵⁻⁷ The glycosylation present on the mAbs must be consistent and human-like to prevent adverse effects.⁵ Therefore, monitoring of IgG glycosylation is essential in cell line and clone selection and during cell culture optimization, where a high number of samples are subjected to product quality analysis including glycosylation profiling, and where fast analysis is required.

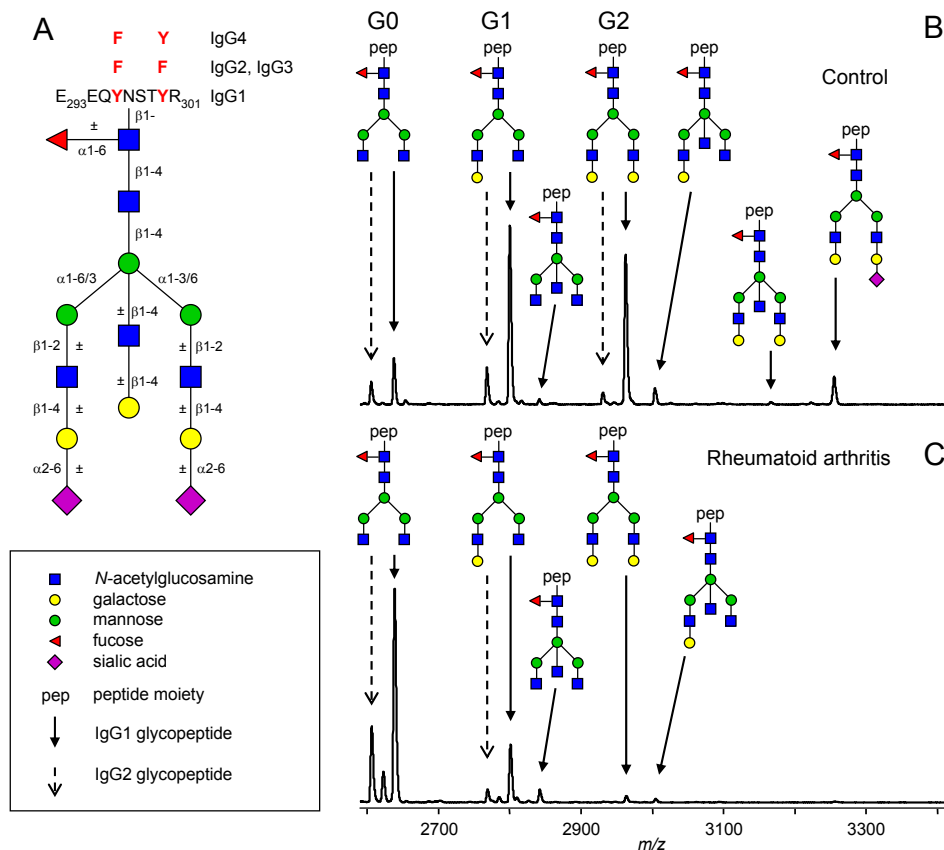


Figure 1. Fc glycosylation monitoring for human IgG at the level of tryptic glycopeptides. (A) The four human IgG subclasses show differences in the peptide sequence and a considerable variation (microheterogeneity) in the structure of the attached N-glycan. The glycosylation profiles of IgG1 and IgG2 tryptic glycopeptides were obtained from Protein A-purified human serum IgG of a control person (B) and a rheumatoid arthritis patient (C) and show pronounced differences in the relative intensities of G0F, G1F and G2F structures. Analyses were performed by MALDI-TOF-MS in the linear positive mode using 2,5-dihydroxybenzoic acid (DHB) as matrix substance.

IgG glycosylation in healthy human populations

Many descriptive data regarding the biological and clinical role of IgG Fc N-glycosylation have been accumulated over the past 30 years. For healthy donors the obtained IgG glycosylation data showed an association of certain IgG glycosylation features with age, sex, and pregnancy. A first publication on the age-dependency of IgG glycosylation was from Parekh *et al.* in 1988,⁸ who studied the degree of IgG N-glycan galactosylation for a group of 151 healthy individuals of both sexes varying in age from 1 to 70 years. The

authors did not distinguish between Fc and Fab glycosylation and focused specifically on galactosylation. All heterogeneity in terms of attached sialic acids, N-acetylglucosamine and fucose was eliminated by performing corresponding exoglycosidase treatments prior to oligosaccharide analysis, leaving galactosylation as the only feature to be analyzed. This revealed that the mean incidence of N-glycans on IgG with both outer arms terminating in N-acetylglucosamine is higher than 30% on average at birth, reaching a minimum of approximately 20% for persons of 25 years of age and then increases continuously to reach approximately 40% by 70 years of age. The mean incidence of monogalactosylated N-glycans remains remarkably constant at approximately 40% over the whole age range, while the digalactosylated N-glycans shows a pattern which is complementary to the parabolic age-dependency curve of the non-galactosylated N-glycans. No sex-specific differences were found. Notably, much of the biological variation observed in this study could not be explained by sex and age heterogeneity.

Approximately 10 years later two IgG glycosylation studies in healthy individuals were published by Japanese research teams. Yamada *et al.* (1997) enzymatically released N-glycans from human serum IgG of 176 female and 227 male individuals ranging in age from 0 to 84 years.⁹ N-glycans were labeled with 2-aminopyridine, desialylated, and profiled by reverse phase high performance liquid chromatography (RP-HPLC) with fluorescence detection, allowing the registration of 12 different glycan structures. This study confirmed the age-dependency of galactosylation⁸ for both sexes. In addition, a difference in the degree of galactosylation was found between males and females at age of approximately 25 years. Women of this age showed on average lower levels of agalactosyl glycoforms than men. Moreover, the occurrence of bisecting N-acetylglucosamine showed an age-dependency. The incidence of bisecting N-acetylglucosamine in healthy individuals generally increased with age and seems to reach a plateau at the age of 50 years.

Corroborating the findings of Yamada *et al.*⁹ Shikita *et al.* (1998) released N-glycans by hydrazinolysis from human serum IgG of a small cohort consisting of 43 female and 37 male individuals ranging in age from 18 to 73 years.¹⁰ N-glycans were labeled with p-aminobenzoic acid ethyl ester (ABEE) and analyzed for their degree of sialylation by anion exchange chromatography. Glycans were profiled by RP-HPLC with fluorescence detection after desialylation, allowing the registration of 12 glycan structures, demonstrating the age-dependency of galactosylation only for females. For the males a slight trend towards lower galactosylation at higher age was observed. However, this tendency was not found to be significant with the small number of males included in the study due to the considerable biological variability. The decreasing incidence of monosialylated N-glycans with age might be explained by the lower incidence of galactosylated antennae (acceptor structures) at higher life-time. The incidence of bisecting GlcNAc was found to increase with age for both sexes.

Recently, the age and sex dependency of IgG galactosylation and bisecting GlcNAc were confirmed in two large scale clinical studies where glycosylation of affinity purified

IgG was studied either in 1821 individuals as released and 2-AA labeled N-glycans by HILIC coupled to fluorescence detection¹¹ or in 1967 individuals as tryptic Fc N-glycopeptides by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS).¹²

A first indication for a particularly high degree of IgG galactosylation during pregnancy was published by Pekelharing *et al.*¹³ In the following, Rook *et al.* showed for ten healthy women and 8 RA patients a low degree of IgG G0F during pregnancy.¹⁴ This was recently confirmed in 148 pregnant RA patients and 32 healthy controls for two IgG subclasses (IgG1 and IgG2),¹⁵ and in a pregnant RA patient for three IgG subclasses (IgG1, IgG2 and IgG4).⁴ In addition, glycosylation of IgG from the fetal circulation was analyzed and compared to maternal IgG.¹⁶ Interestingly the incidence of IgG G0F was found to be approximately 25% higher in maternal serum as compared to fetal umbilical vein serum, which might indicate a glycosylation-sensitive transport of maternal IgG via the placenta to the fetal circulation with a selection for highly galactosylated glycoforms. Alternatively, this difference may be caused by the difference in the relative abundance of IgG subclasses in umbilical vein serum as compared to maternal serum.¹⁷

In conclusion, glycosylation of IgG varies considerably between individuals of the same sex and age. The differences between sexes are minor compared to the influence of life-time. There is a clear age-dependency of IgG N-glycan galactosylation and decoration with bisecting GlcNAc. Galactosylation increases after birth up to age 25 years and decreases again with higher life-time. This decrease in galactosylation seems to be accompanied by a decrease in sialylation. Moreover, pregnancy is associated with a temporal increase in IgG galactosylation.

IgG glycosylation in diseases

In 1985, researchers from Oxford and Tokyo showed an increase in IgG G0F for patients with RA and osteoarthritis³ and many more reports have followed describing similar IgG glycosylation features for other autoimmune diseases, infectious diseases, and cancer. An overview of key publications on diseases with an elevated incidence of G0F on IgG is given in Table 1. Low galactosylation of IgG is clearly a common feature in various diseases. In RA, IgG G0F has been shown to correlate with clinical parameters like joint destruction and disease activity and may serve as an indicator for the disease course.¹⁸ Interestingly, remission of arthritis is often observed during pregnancy, which is associated with a decrease of IgG G0F.¹⁴ A pathogenic role of IgG G0F autoantibodies has been demonstrated by Rademacher *et al.* for murine collagen-induced arthritis,¹⁹ where treatment of an autoantibody-containing IgG preparation with β -galactosidase to increase the level of IgG G0F glycoforms resulted in a more efficient induction of arthritis than the untreated IgG preparation. In the following, a role has been suggested for the mannose-binding protein in complement activation by IgG G0F glycoforms in

RA.²⁰ However, to establish the mechanisms of pathogenicity of IgG G0F in RA and related diseases more research will be required.

The hitherto studied autoimmune diseases showing lowered IgG galactosylation are characterized by both cell-mediated and humoral autoimmunity. The study of IgG glycosylation in antibody-mediated autoimmune diseases such as Lambert-Eaton myasthenic syndrome (LEMS) and myasthenia gravis (MG) for which the role of

Table 1. Diseases for which elevated IgG G0F and/or lowered IgG galactosylation have been reported

Disease	Study cohort	Analytical method	Reference
rheumatoid arthritis (RA)	19 patients	glycan release and chromatography	3
RA	127 patients	lectin binding assay	18
RA	29 patients	lectin binding assay	21
RA	232 patients	glycan release and chromatography	22
RA	29 patients	lectin binding assay	23
RA	85 patients	glycopeptides and mass spectrometry	24
RA	148 patients	glycopeptides and mass spectrometry	15
juvenile chronic arthritis	34 patients	lectin binding assay	25
adult onset RA	53 patients	glycan release and chromatography	26
juvenile onset RA	27 patients	glycan release and chromatography	26
juvenile onset RA	9 patients	lectin binding assay	21
Osteoarthritis	13 patients	glycan release and chromatography	3
Osteoarthritis	13 patients	lectin binding assay	21
Crohn's disease	9 patients	glycan release and chromatography	27
Crohn's disease	17 patients	lectin binding assay	21
systemic lupus erythematosus with Sjögren's syndrome	10 patients	glycan release and chromatography	27
systemic lupus erythematosus	12 patients	lectin binding assay	21,28
ankylosing spondylitis	12 patients	lectin binding assay	21
ulcerative colitis	32 patients	immunoassay with anti-GlcNAc mAb	29
ulcerative colitis	16 patients	lectin binding assay	21
infective endocarditis	12 patients	lectin binding assay	21
Tuberculosis	21 patients	glycan release and chromatography	27
liver fibrosis and cirrhosis in hepatitis C infection	~200 patients	lectin binding assay (AAL; alauria aurantia lectin) ¹	30
HIV-infection	28 patients	lectin binding assay; glycan release and mass spectrometry	31
ovarian cancer	3 patients	glycan release and chromatography	32
Myositis	14 patients	glycopeptides and mass spectrometry	33

¹ specific analysis of anti- α -Gal antibodies.

pathogenic antibodies against well known target antigens has been described in detail could provide valuable insights into the influence of IgG Fc N-glycosylation features on antibody pathogenicity.

A few reports have shown changes in IgG galactosylation during a humoral immune response. For example, Lastra *et al.* have shown in mice that during a humoral immune response IgGs with a low degree of galactosylation are produced, whereas, antibodies became more highly galactosylated when IgG titers were falling.³⁴ Moreover, Kaneko *et al.* found a specific decrease of IgG sialylation during humoral immune response in a mouse nephropathy model.³⁵ Future studies will hopefully shed light on the regulation and dynamics of IgG glycosylation in human humoral immune response.

Antibody-dependent cellular cytotoxicity

IgG Fc N-glycosylation is crucial for high-affinity interaction with Fc γ -receptors, which can be demonstrated by enzymatically removing parts of the glycan structures. After cleavage of the chitobiose core of the N-glycan by endoglycosidase Endo S from *Streptococcus pyogenes* (leaving a single N-acetylglucosamine on the Fc portion),^{36,37} IgG binding to the Fc γ -receptor changes and the binding to activated monocytes decreases. Endo S was found to have a protective effect in a mouse model of idiopathic thrombocytopenic purpura and is a potential therapeutic agent against antibody-mediated autoimmune diseases.³⁸ Notably, Fc N-glycans influence the binding to Fc γ -receptors by determining the conformational structure of the Fc moiety. Fully galactosylated N-glycans are positioned between the Fc polypeptide chains, resulting in an open Fc conformation. Removal of N-glycans resulted in a closed conformation, which is unfavorable for most IgG Fc γ -receptor interactions.³⁹

Next to the removal of most of the Fc N-glycans by Endo S treatment, more subtle changes in the IgG Fc glycosylation pattern, as achieved by choosing different expression systems⁴⁰ and by modifying the glycosylation using exoglycosidases,^{41,42} have likewise been shown to have a profound influence on the interaction of the IgG Fc portion with Fc γ -receptors. Many reports have shown that the presence or absence of core-fucose is the most important feature with respect to antibody-dependent cellular cytotoxicity (ADCC) of IgG1, while the degree of sialylation, antenna galactosylation, and the presence of bisecting N-acetylglucosamine do not seem to have a strong influence.⁴¹⁻⁴⁴ The lack of core-fucoses on IgG1 Fc N-glycans has been shown to result in an enhanced binding to Fc γ -receptor IIIa expressed on macrophages leading to a drastic enhancement of ADCC.^{43,44} Enhanced ADCC activity of afucosylated IgG1 may have profound effects on the severity of antibody-mediated autoimmune diseases.⁴³⁻⁴⁵ In *in-vitro* ADCC assays antibodies lacking core-fucosylation have shown enhanced efficacy.^{44,46} Glycosylation of therapeutic antibodies is, therefore, often engineered to achieve lower core-fucosylation and high ADCC activity.^{5,46}

Intravenous immunoglobulin

Glycosylation of IgG has recently received much attention due to the findings of Ravetch and coworkers,^{35,47} who analyzed the protective effect of intravenous immunoglobulin (IVIg) in a mouse arthritis model and could demonstrate that only the Fc parts exhibit the immunosuppressive function. Moreover, using both neuraminidase treatment and enrichment of sialylated species by lectin affinity chromatography, they showed that sialylated IgGs as well as sialylated IgG Fc moieties are much more potent in preventing pathology.³⁵ In a mouse model, the 2,6-sialylated N-glycan antennae on the Fc moieties were identified to be the motifs recognized by the C-type lectin SIGN R1 (an orthologue of the human Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin; DC-SIGN) expressed on murine macrophages in the splenic marginal zone, thereby inducing the expression of inhibitory IgG Fc γ -receptors on effector macrophages.^{47,48} In a humanized DC-SIGN mouse model the same group revealed that administration of 2,6-sialylated Fc moieties suppressed inflammation through a novel T_H2 pathway via production of the cytokine IL-33.⁴⁹ Recently, Stadlmann *et al.*⁵⁰ refined the study on anti-inflammatory properties of Sambucus nigra agglutinin (SNA) lectin enriched IVIg and argued that the anti-inflammatory properties of IVIg accounts from double sialylation of IgG.^{50,51} Contradictory results were obtained for the SNA fraction of IVIg by Guhr *et al.*⁵² who observed only enrichment of Fab sialylated IgG. Moreover, the SNA fraction was shown to have no effect on the platelet count in a murine model of passive immune thrombocytopenia, suggesting that SNA lectin is not a suitable method to enrich Fc sialylated IgG.

Refinement of the receptor binding properties of IgG Fc moieties and derived recombinant drug candidates is expected to provide a way of efficient treatment of autoimmune disorders.^{47,53} Hitherto IVIg has been used on the basis of its anti-inflammatory properties at high doses (above 1-2 g/kg) for the treatment of various autoimmune diseases such as RA, idiopathic thrombocytopenic purpura, systemic lupus erythematosus, and Guillain-Barré syndrome.^{54,55}

Regulation of IgG glycosylation

B-lymphocytes may produce distinct IgG Fc glycosylation profiles. For example, Fc N-glycans of antigen affinity purified IgG1 thought to be involved in the pathogenesis of rheumatoid arthritis have been shown to harbor different glycoforms compared with total sera IgG1.^{24,45,56} Furthermore, the glycosylation of IgG1 produced by B lymphocytes *in-vitro* has been shown to be influenced by environmental factors and factors known to stimulate the innate or adaptive immune system.⁵⁷ *In-vitro* stimulation of B-cells with all-trans retinoic acid (environmental factor) resulted in the expression of IgG1 with decreased galactosylation within a time-range of several days, while increased galactosylation and reduced bisecting GlcNAc have been observed after stimulation

with CpG oligodeoxynucleotide (stimulates the innate immune system) or interleukin 21 (stimulates the adaptive immune system).⁵⁷

IgG stability and half-life

The absence of Fc N-glycosylation on IgG does not affect antigen binding affinity,^{58,59} but it has been demonstrated with monoclonal antibodies that the structure of the C_H2 domain is altered resulting in decreased stability.^{41,42,60-62} In a pharmacokinetics study in humans and cynomolgus monkeys, the N-glycan composition in the Fc domain of human recombinant p55 tumor necrosis factor receptor-immunoglobulin G1 fusion protein (Lenercept) was shown to be drastically changed during the first 24 h after injection after which the glycosylation remained constant over 10 days.⁶³ In the rapid initial phase it was observed that therapeutic protein complexes carrying Fc N-glycans with terminal GlcNAc were rapidly cleared suggesting clearance by the mannose receptor. Similar reports have, however, not yet been presented for IgG. In the human circulation increased clearance has been shown for therapeutic IgGs containing high-mannose glycans.⁶⁴ Although the quantitative effect on pharmacokinetic area under the curve was shown to be relatively minor it strongly emphasizes that the high mannose content may affect pharmacokinetic properties of therapeutic antibodies and should be considered an important product quality attribute. Likewise, Chen *et al.* observed a decrease of Fc N-mannosylation for IgG2 in a human pharmacokinetic study. Intriguingly, they found similar changes after *in-vitro* incubation of the antibody in serum, suggesting high mannose glycan cleavage instead of clearance.⁶⁵ More studies are required to allow the elucidation of the effect of Fc N-glycan composition on the IgG clearance.

IgG purification

Parekh *et al.* (1985) were the first to analyze IgG glycosylation in great detail.³ To purify the IgGs, ammonium sulphate precipitation and anion-exchange chromatography was used, based on a protein fractionation protocol which is considerably older.⁶⁶ Adaptations of this method are still in use, primarily for the purification and subsequent glycan release of IgG from human serum or plasma.^{10,67-69} The most popular technique is, however, affinity-purification using immobilized Protein A (e.g.⁷⁰⁻⁷³) and Protein G (e.g.^{9,74-76}). These bacterial proteins bind to IgGs of various species and are commercially available in immobilized form (e.g. Sepharose beads and magnetic beads). IgGs may be loaded at neutral pH and elution is achieved at low pH. Generally, Protein A and Protein G columns are reusable for many purification cycles. The use of Protein A and Protein G is particularly popular for the purification and subsequent glycosylation analysis of recombinant mAbs. For the purification of IgG from human serum, both Protein A and Protein G may be applied, with the important difference that Protein

G will bind all 4 human IgG subclasses, while Protein A will only retain IgG1, IgG2, and IgG4, but not IgG3.^{4,77} Additional sample preparation steps like buffer exchange or desalting may be necessary to get the IgG samples purified for downstream analytical methods.⁷⁸

An alternative approach for IgG purification from plasma or serum is the use of SDS-PAGE, followed by the in-gel N-glycan release or generation of glycopeptides.⁷⁹ Moreover, the simultaneous glycosylation analysis of major human serum glycoproteins after albumin depletion has been demonstrated by RP-HPLC-MS/MS at the glycopeptide level, covering the glycosylation profiles of 23 N-glycosylation sites of serum glycoproteins including the Fc part of the different IgG subclasses.⁸⁰

Released N-glycans

Starting from purified IgG, glycans are released using either enzymatic (e.g. N-glycosidase F; PNGase-F)^{72,75,81,82} or chemical (e.g. hydrazinolysis)^{10,69,83,84} cleavage. PNGase-F releases all asparagine-linked oligosaccharides with high yields, unless they are α 1-3 core-fucosylated (core-modification not observed in mammals, but common in plants). Hydrazinolysis allows the release of O-glycans in addition to N-glycans. However, hydrazinolysis is a rather harsh method leading to the loss of N-acetyl groups that are present on N-acetylglucosamine residues of the N-glycans thus requires re-N-acetylation. Moreover, working with anhydrous hydrazine requires special safety precautions, and any contact with water has to be avoided.⁸⁵

Three major strategies are used for analyzing oligosaccharides from glycoproteins such as antibodies. A first approach includes labeling of the reducing end of released glycans (e.g. 2-aminobenzoic acid, 2-aminobenzamide)⁸⁶⁻⁸⁸ followed by liquid chromatography (e.g. reverse phase,^{89,90} hydrophilic interaction)^{11,32,75,91,92} or capillary electrophoresis.⁹³⁻⁹⁵ These separation techniques may be combined with fluorescence detection or mass spectrometry. Second, native reducing-end N-glycans may be analyzed by MALDI-TOF-MS,^{78,96} high-pH anion exchange chromatography with electrochemical detection,⁹⁷ or by microfluidic chip LC-MS.⁹⁸ Third, permethylation of all hydroxyl groups (stabilizes the sialic acids) may be performed, followed by mass spectrometric analysis using either MALDI or (microchip)LC-ESI-MS.^{77,99} With these methods, the composition of the glycans can be established. Even though mass spectrometric methods for the analysis of released native or labeled glycans comprise generally highly sensitive procedures, no information can be obtained regarding linkages, epimericity, and anomericity. In order to obtain this information, several studies have applied multiple exoglycosidases.^{78,83,93,96} Furthermore, unambiguous assignment of glycans to the Fc and Fab moieties can be achieved when antibody fragments are separation prior to N-glycan release.^{5,72,100,101}

Glycopeptides

Various specific and nonspecific proteolytic enzymes are available for the digestion of glycoproteins, including trypsin,^{102,103} lysylendopeptidase (Lys-C),¹⁰² endoproteinase Glu-C (V8 protease)¹⁰⁴ and pronase.¹⁰³⁻¹⁰⁵ In general, completeness of proteolytic cleavage is improved by the addition of denaturing agents, such as guanidine hydrochloride, urea and acetonitrile. In addition, reduction of cysteine disulfide bonds by reducing agents such as dithiothreitol (DTT) and β -mercaptoethanol further improves accessibility of proteolytic cleavage sites. Since not all proteolytic enzymes retain their full activity under reducing conditions, and in order to avoid reformation of disulfide bonds during (glyco-)peptide storage and analysis, sulfhydryl groups are often blocked with alkylating agents, such as iodoacetamide, iodoacetic acid and ethyleneimine. To ensure full activity of the proteolytic enzyme, the reduction/alkylation buffer is usually diluted or replaced by a digestion buffer using dialysis and gel filtration which may be followed by lyophilization of the desalted sample.^{80,106,107} In order to simplify IgG analysis, heavy and light chains resulting from the reduction and alkylation can be separated by RP-HPLC or SDS-PAGE prior enzymatic digestion. This approach is often applied in studies characterizing recombinant monoclonal antibody glycosylation.^{96,108,109} It should be taken into account that peptides with a N-terminal glutamine residue or a carboxyamidomethylated cysteine (product after alkylation with iodoacetamide) often reveal additional (glyco)peptides with ammonium loss (-17 Da) due to pyroglutamic acid or 5-oxothiomorpholine-3-carboxylic acid (cyclization of the carboxyamidomethylated cysteine) formation, respectively.¹¹⁰

An improvement of the sample-processing using proteolytic cleavage reactions is often achieved by the use of enzymes which are immobilized on beads, monoliths, or on capillary walls, offering the advantage of enzyme re-usability, automation and complete digestion in very short times with a resulting peptide mixture lacking contamination with protease and its auto-proteolysis fragments.¹¹¹⁻¹¹⁵

Trypsin (serine protease) is by far the most widely applied proteolytic enzyme in proteomics and glycoproteomics, cleaving peptides at the carboxyl side of lysine and arginine, unless the following amino acid is a proline. IgG Fc glycopeptides resulting from tryptic digestion are often easy to ionize by ESI and MALDI. Trypsin digestion is typically performed overnight at 37°C in a 10 to 100 mM reaction buffer (Tris-HCl or ammonium bicarbonate, pH~8) using a 1:100 to 1:20 protease:protein ratio.^{4,80,92,96,102,113,116-118} To improve trypsin digestion enzyme mixtures¹⁰² or denaturing agents such as guanidine-HCl or urea with concentrations up to 2 M are sometimes applied.^{96,113,119} Interestingly, addition of acetonitrile with concentrations up to 80% has been shown to improve enzymatic activity and digestion efficiency for a protein standard mixture,^{120,121} though this has not been shown for IgG.

Samples prepared by trypsin digestion have successfully been used for the identification and characterization of the glycosylation of mAbs^{96,118,122} and serum

IgG.^{4,79,80,117,123} Due to the occurrence of tryptic Fc glycopeptides with identical peptide moieties for the IgG2 and IgG3 subclasses, the analysis of a proteolytic digest of total serum IgG will not allow the specific assignment of glycopeptides to these subclasses.⁴ Therefore, serum IgG was fractionated into a pool of IgG1, IgG2, and IgG4 by Protein A capturing, followed by the capturing of IgG3 from the flow-through using Protein G beads. Analysis of the trypsinized eluates by RP-HPLC allowed the unambiguous assignment of the observed tryptic glycopeptides to specific IgG subclasses.^{4,45,56}

Mass spectrometry of glycopeptides

Mass spectrometric detection of glycopeptides offers the possibility to determine glycosylation profiles in a site-specific manner. Traditionally, glycopeptides were ionized by fast atom bombardment and laser desorption. Within the past two decades, the softer ionization techniques electrospray ionization (ESI) and MALDI came in place, providing a higher sensitivity and making intact ions of high-molecular mass compounds accessible.

MALDI of tryptic IgG glycopeptides typically results in singly charged $[M+H]^+$ ions,^{92,96,102,116,117,119,124} while ESI often results in doubly charged $[M+2H]^{2+}$ ions^{4,80,107,125} and/or triply charged $[M+3H]^{3+}$ ions.^{4,71,79} However, quadruply charged $[M+4H]^{4+}$ ions due to one missed cleavage (see Figure 2C in⁷⁹) and doubly charged sodiated $[M+H+Na]^{2+}$ ions¹¹⁶ of tryptic IgG glycopeptides have also been reported with ESI. The ESI conditions markedly influence the charge state distribution, with harsher ion transfer conditions leading to lower charge states.

Ionization of glycopeptides can be suppressed by highly abundant coexisting peptide ions. In addition, salts present in the original sample or introduced during reduction, alkylation and enzymatic cleavage can suppress glycopeptide ionization, especially with ESI. Finally, overlapping isotope clusters from coexisting peptides or missed cleavages can complicate glycopeptide mass determination and identification. Therefore, proper sample pretreatment (enrichment, efficient enzymatic cleavage, desalting) prior to ionization is important. Sample purification and pre-separation of (glyco)peptides can be achieved using hyphenated techniques such as LC-ESI-MS. Alternatively, samples can be fractionated and desalted by preparative LC or SPE, followed by MALDI-MS.

RP-HPLC-ESI-MS

One of the most widely applied analytical techniques for glycopeptide analysis involves direct analysis of proteolytic digests by RP-HPLC coupled to ESI-MS. RP-HPLC separation of proteolytic IgG digests is mostly performed on C18 analytical columns of various dimensions. As an alternative to RP-HPLC, graphitized carbon chromatography can be applied.¹²⁶ In general, a water/acetonitrile gradient with increasing acetonitrile concentrations, typically containing approximately 0.1% acidic mobile phase additive

such as trifluoroacetic acid (TFA) or formic acid (FA) is used in RP-HPLC-MS.^{4,80,106,107,127} TFA is a commonly used mobile phase additive due to its positive effect on analyte retention and peak concentration.¹²⁸ However, TFA can form gas-phase ion-pairs with positively charged analyte ions potentially increasing ESI suppression.^{128,129} For IgG glycosylation analysis, FA is therefore applied more often as acidic mobile phase additive. In addition, to ensure method stability in terms of separation, ionization and detection, most studies have applied micro-LC^{107,127,130} or capillary-LC^{79,80,106,109} coupled to ESI-MS, while nanoLC is less frequently used.^{4,45,56,71,125,131}

ESI-MS/MS spectra of LC separated glycopeptides can be high-lighted on the basis of glycan specific oxonium ions of m/z 204 [HexNAc+H]⁺, 366 [Hex₁HexNAc₁+H]⁺, 528 [Hex₂HexNAc₁+H]⁺, and 657 [Hex₁HexNAc₁NANA₁+H]⁺ (Hex, hexose; HexNAc, N-acetylhexosamine; NANA, N-acetylneuraminic acid) obtained on collision induced dissociation (CID) or by scanning for specific neutral losses of terminal monosaccharides such as fucose, N-acetylglucosamine, and sialic acid.^{80,116,132} Moreover, multiply charged IgG glycopeptide ions as generated by ESI allow the performance of CID and electron transfer dissociation (ETD) experiments, with the latter resulting in peptide sequence information.⁴ Glycopeptides with a common peptide moiety generally elute close to each other. Interestingly, some RP-HPLC columns show an enhanced retention of sialylated glycopeptides when compared to glycopeptides with neutral glycan chains,⁴ while other RP-HPLC columns do not exhibit this pronounced difference in retention times.⁷⁹ To ensure the measurement of intact glycopeptides, in source decay, which often results in the loss of sialic acid or the cleavage of the GlcNAc β 1-2Man linkage, has to be ruled out by choosing the ion transfer voltages carefully.⁷⁹ This allows the acquisition of Fc glycosylation profiles by RP-HPLC-ESI-MS,^{79,80,133,134}

Mass spectrometry of glycopeptides allows the determination of glycan structure and attachment site in a single experiment. For example, analysis of lysyl endopeptidase-digested mouse antidansyl mAb IgG2b (RCM-IgG2b) by an automated LC-MS(/MS) mode, allowed assignment of twelve different types of O-linked oligosaccharides to the heavy chain threonine 221 in the hinge region.¹⁰⁶ Moreover, three major types of N-linked oligosaccharides could be assigned to the heavy chain asparagine 297 from endoproteinase Glu-C digested RCM-IgG2b (see Figure 5C in¹⁰⁶).

Accurate MS measurement of glycopeptide amounts in complex samples is complicated by ionization variability. UV-detection, by contrast, often allows proper quantification in peptide mapping by RP-HPLC.¹⁰⁸ Due to the unspecific nature of UV detection, quantification is only possible when compounds are at least partially resolved into individual LC peaks. Monitoring LC separations with in-line UV followed by ESI-MS characterization and identification combines the quantitative strength of UV with the profiling strength of MS. Additionally, an extra quality control step is offered to determine digestion completeness, separation quality and sample identity.

As for IgG glycosylation, quantification of non-glycosylated, deglycosylated and total N-glycosylated species in tryptic digests of Herceptin IgG1 was possible by RP-

UPLC-UV-MS.¹³⁵ Non-glycosylated and deglycosylated peptides were separated into individual LC peaks. The total N-glycosylated species were determined by the analysis of the tryptic digests before and after PNGase-A treatment. Additionally, IgG1 purified from human polyclonal IgG was analyzed, showing the presence of a peptide co-eluting with the deglycosylated peptides. This contamination prevented proper quantification on the basis of UV only, pointing out the necessity of combination with the more tedious and time-consuming MS detection.

HILIC-ESI-MS

HILIC coupled to ESI-MS is another powerful technique for the separation and analysis of glycopeptides. While retention in RP-HPLC is primarily mediated by hydrophobic interactions of the peptide moiety, retention in HILIC is mainly achieved via hydrophilic interactions between the stationary phase and the glycan moieties of the glycopeptides, which results in the (partial) separation of glycopeptides from peptides.^{92,136-140} Retention will generally increase with the size of the glycan moiety. Therefore, separation of glycopeptides can be achieved according to the degree of sialylation.¹⁴⁰⁻¹⁴² ZIC-HILIC separation of tryptic IgG1 and IgG2 glycopeptides containing the same glycan moiety showed that the presence of more hydrophilic amino acids in the peptide chain affects glycopeptide retention.⁹² Tryptic IgG1 Fc glycopeptides which contain two tyrosine residues showed more retention than tryptic IgG2 Fc glycopeptides which instead contain two phenylalanine residues, thus structurally only differing by two aromatic hydroxyl groups. Moreover, with ZIC-HILIC it was possible to separate IgG glycopeptides with identical peptide moieties containing isomeric glycan structures. For example, G1F glycopeptides of both IgG1 and IgG2 containing an upper antenna galactose were efficiently separated from those containing a lower antenna galactose.⁹² Similar separation of upper and lower antenna galactosylation was recently shown for a monoclonal IgG1 using an ACQUITY BEH HILIC column.¹³⁶

Detailed glycan structural information can be obtained by sequential exoglycosidase treatments of antibody glycopeptides followed by LC-MS. For example, sequential exoglycosidase treatments of endoproteinase Lys-C digested humanized anti-A β IgG1 monoclonal antibody showed that the major N-linked oligosaccharide structures in the Fv region were core-fucosylated biantennary oligosaccharides with no or one β -linked N-acetylgalactosamine, zero or one α -linked galactose and one or two N-glycolylneuraminic acid residues.¹³⁴ In addition, typical core-fucosylated, biantennary N-glycans with one or two galactose residues were observed in the Fc region.

MALDI-MS

IgG glycosylation is often studied by MALDI-MS. Generally, MALDI is less sensitive to ion suppression than ESI. Nevertheless, direct glycopeptide analysis from proteolytic

digests is often prohibited by the presence of non-volatile salts, detergents and non-glycosylated peptides. In addition, occurrence of overlapping isotope clusters from coexisting peptides or missed cleavages can further complicate glycopeptide identification and mass determination. Therefore, IgG glycopeptides are often enriched and desalted by RP-HPLC fractionation,^{96,117,119,124} HILIC affinity chromatography,^{102,119,143} or lectin-based affinity chromatography.¹¹⁹ MALDI sources are most often coupled to time-of-flight (TOF) MS, but also to ion trap-MS, FTICR-MS, and Orbitrap MS.

MALDI-TOF-MS analysis of tryptic IgG glycopeptides overlaid, mixed or sandwiched with 2,5-dihydroxybenzoic acid (DHB) and/or α -cyano-4-hydroxycinnamic acid (CHCA) can be performed in linear positive, linear negative, reflectron positive, and/or reflectron negative mode. Due to the soft nature of MALDI ionization most IgG glycopeptide ions remain intact. Sialylated glycopeptides, however, when analyzed by positive-ion or negative-ion MALDI-TOF-MS with delayed extraction in the reflectron mode, often show massive desialylation due to in-source decay and metastable decay caused by laser-induced decomposition and/or collisional fragmentation during ion acceleration. By contrast, detection of sialylated glycopeptides can be achieved by MALDI-TOF-MS in the linear mode,^{102,119} since the lack of an extraction delay strongly reduces in-source decay of the sialylated glycopeptides. Moreover, any metastable decay products formed after ion acceleration are not separated from their precursor ions in linear mode.

Large transfer voltages as applied in TOF instruments require MALDI to be performed under high vacuum. By contrast, trapped-ion mass spectrometers allow the use of high pressure MALDI sources which demonstrated the ability to cool the internal vibrational energy of ions and allowed registration of intact labile molecules such as sialylated gangliosides.¹⁴⁴

Additional structural information can be obtained by CID if MALDI is coupled to tandem mass spectrometers, such as QIT/TOF, QTOF, and TOF/TOF mass spectrometers. For example, CID of $[M+H]^+$ glycopeptide ions by MALDI-QIT/TOF allowed glycan identification and sequencing.¹⁰² In addition, fragment ions corresponding to the peptide with attached N-acetylglucosamine were formed, which could be isolated and subjected to CID providing peptide sequence and glycan attachment site information in MS³. Notably, MALDI-TOF/TOF-MS as well as MALDI-QTOF-MS analyses of glycopeptides often directly provide detailed peptide sequence information. In contrast, product ion spectra obtained by automatic LC-MS, LC-MS/MS often lack sufficient information, as exemplified for a glycosylated peptide.¹⁴⁵

Scope

In the pharmaceutical industry the detailed characterization of the glycans on biopharmaceuticals (e.g. therapeutic monoclonal antibodies) is of outmost importance to prevent detrimental side effects and ensure product quality. Detailed characterization of polyclonal IgG glycosylation changes as part of humoral immune responses could provide valuable information on the regulatory mechanisms involved. Two potential strategies to determine IgG Fc N-glycosylation are the analysis of released N-glycans or glycopeptides by mass spectrometry. While valuable information regarding N-glycan heterogeneity is provided with both strategies, only IgG glycopeptide analysis allows discrimination between different IgG subclasses and provides N-glycan profiles that are Fc specific. The scope of this thesis was to develop miniaturized (high-throughput) sample preparation and analysis strategies for the more detailed characterization of IgG Fc N-glycosylation by mass spectrometry. The methods should be applicable to characterize the glycosylation of therapeutic monoclonal antibodies as well as human polyclonal IgG and had to be applied for clinical cohorts to gain insights into the mechanisms regulating IgG Fc glycosylation. In clinical glycomics high-throughput analysis with low sample amounts is often mandatory, as large cohorts result in a high statistical power and biological material is precious.

In **Chapter 2** the development of a fast and robust nanoLC-MS method for the analysis of human polyclonal IgG Fc N-glycopeptides is presented. A thorough method validation and evaluation of pregnancy related IgG Fc N-glycosylation changes are included.

Chapter 3 describes the development of cotton HILIC SPE microtips for micro-scale purification and enrichment of glycopeptides and released glycans. Furthermore, the potential of the microtips for analyzing neutral and sialylated glycoconjugates as well as the capacity of the cotton stationary phase is shown.

In **Chapter 4** the development of a high-throughput IgG Fc N-glycopeptide profiling method by MALDI-FTICR-MS is described. Moreover, the potential of the developed method for clinical applications is shown with samples from presumably healthy individuals.

In **Chapter 5** the rationally designed 4-chloro- α -cyanocinnamic acid (Cl-CCA) matrix is evaluated for MALDI-TOF-MS analysis of sialylated glycoconjugates. The performance of the matrix is shown for glycopeptides and released glycans from various proteins in positive and negative-ion mode.

Chapter 6 describes the application of the developed fast and robust nanoLC-MS method for the evaluation of IgG Fc N-glycosylation changes in Caucasian adults and African children upon vaccination. IgG glycosylation changes in total plasma IgG as well as antigen-specific purified IgG are evaluated.

Chapter 7 describes the application of the developed high-throughput MALDI-FTICR-MS IgG Fc N-glycopeptide profiling method for the evaluation of Fc N-glycosylation changes in Lambert-Eaton Myasthenic Syndrome and myasthenia gravis.

Finally, a general discussion is given in **Chapter 8**.

Fc specific IgG glycosylation profiling by robust nano-reversed phase HPLC-MS using a sheath-flow ESI sprayer interface

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Abstract

Biological activities of immunoglobulin G such as effector functions via Fc γ -receptor interactions are influenced by Fc linked N-glycans. Here we describe a fast, robust and sensitive nanoLC-ESI-MS method for detailed subclass-specific analysis of IgG Fc N-glycosylation. High capacity extraction and fast separation of tryptic (glyco) peptides from affinity chromatography purified human polyclonal plasma/serum IgG was achieved by combining a porous particle C18 trap column with a fused core particle C18 nanoLC column. A sheath-flow ESI sprayer was used as a sensitive zero dead volume plug-and-play interface for online MS coupling, generating a very constant spray and ionization over the entire LC gradient. The propionic acid containing sheath-liquid effectively suppressed TFA gas-phase ion-pairing, enabling the use of TFA containing mobile phases. The fixed position of the sheath-flow ESI sprayer, far away from the glass capillary inlet, reduced MS contamination as compared to conventional nano-ESI. The method was found to be suitable for fast and detailed subclass-specific IgG Fc N-glycosylation profiling in human plasma. The obtained subclass-specific IgG Fc N-glycosylation profiles were processed automatically using in house developed software tools. For each of the IgG subclasses the 8 major glycoforms showed an interday analytical variation below 5%. The method was used to profile the IgG Fc N-glycosylation of 26 women at several time points during pregnancy and after delivery, revealing pregnancy-associated changes in IgG galactosylation, sialylation and incidence of bisecting N-acetylglucosamine.

Introduction

In human blood IgG represents the most abundant antibody class (approximately 10 mg/ml serum).^{1,2} IgGs occur in four subclasses (i.e. IgG1-4)⁴ and consist of two heavy and two light polypeptide chains each having a variable and a constant region. The two light chains and parts of the two heavy chains form the fragment antigen binding (Fab) moieties, while the remainder of the two heavy chains forms the fragment crystallizable (Fc) moiety. A biantennary complex type N-glycan is present on each of the heavy polypeptide chains at asparagine 297 in the C_H2 domain of the Fc moiety. This N-glycan is often core-fucosylated and varies in the number of antenna galactose residues resulting in the prominent glycoforms G0F (no galactose), G1F (1 galactose), and G2F (2 galactoses). A small part of the Fc N-glycans may carry a bisecting N-acetylglucosamine (GlcNAc). In addition, these glycans may contain a sialic acid residue attached to an antenna galactose.

Fc N-glycans influence the biological activity of IgG by modulating Fc γ -receptor binding.^{19,35,38,39,43,44,146} For example, cleavage of the chitobiose core with endoglycosidase from *Streptococcus pyogenes* (Endo S) results in a closed Fc conformation which strongly decreases binding to activated monocytes.^{38,39} Even small changes in the Fc N-glycan structure may have profound effects on the Fc γ -receptor binding. The absence of a core-fucose on IgG1 Fc N-glycans was found to promote binding to Fc γ -receptor IIIa expressed on macrophages and resulted in a drastic enhancement of antibody-dependent cellular cytotoxicity (ADCC).^{43,44} Bioengineered antibodies lacking a core-fucose may, therefore, represent a potent new generation of anti-cancer therapeutics.^{44,46} In a mouse arthritis model it has been shown that the anti-inflammatory properties of intravenous immunoglobulin depend on α 2,6-linked sialic acid on IgG Fc N-glycans which lead to Fc mediated signaling via the C-type lectin SIGN R1 (an orthologue of the human DC-SIGN) expressed on murine macrophages in the splenic marginal zone.^{35,47,48} Moreover, decreased levels of IgG Fc sialylation have been observed in a murine nephritis model suggesting that the decrease in sialylation may provide a switch from the steady state innate anti-inflammatory activity to an adaptive pro-inflammatory response.³⁵ For a variety of autoimmune diseases, infectious diseases and cancer lowered IgG Fc galactosylation has been observed.^{3,32,147} Moreover, changes in IgG Fc N-glycans of healthy individuals have been associated with age, gender and pregnancy.⁸⁻¹⁰

Accurate analysis of IgG Fc N-glycosylation is essential for e.g. the characterization of biotechnologically produced therapeutic monoclonal antibodies and obviously is of benefit for the analysis of glycosylation changes of polyclonal IgGs as part of humoral immune responses. Fc N-glycosylation of IgGs can be determined by mass spectrometry (MS) after enzymatically releasing the N-glycans or after preparation of (glyco)peptides. With released N-glycans subclass-specific Fc N-glycosylation profiling is only allowed when IgG subclasses are separated prior to N-glycan release. By contrast, analysis

of IgG Fc N-glycopeptides allows discrimination between subclasses on the basis of the peptide moieties and masses. Analysis of IgG glycopeptide microheterogeneity can be achieved by MALDI-MS^{77,102,119,148} or LC-MS.^{4,50,79,140} MALDI-MS is very well suited for high-throughput analysis and has been shown to allow subclass-specific Fc N-glycopeptide profiling of human polyclonal IgG.¹⁴⁸ However, the presence of isomeric glycopeptides prevents proper determination of IgG2 fucosylation and IgG4 glycosylation.¹⁴⁸ By performing an LC separation prior to MS detection detailed characterization of IgG subclass-specific Fc N-glycosylation can be achieved in a single analysis.^{4,79}

To allow direct coupling of the chromatographic separation with the mass spectrometer, formic acid (FA) is often applied as acidic mobile phase additive.^{45,71,125} Analyte retention and peak shapes can be improved by using trifluoroacetic acid (TFA) in stead of FA.¹²⁸ Moreover, ionic interactions of basic analytes with residual silanol groups on silica based RP stationary phases are strongly reduced with TFA.¹²⁹ However, TFA is known to form gas-phase ion-pairs with positively charged analyte ions resulting in ESI ion suppression. By applying a mixture of isopropanol (IPA) and propionic acid (PA) or acetic acid (TFA fix) prior to ESI this effect can be substantially reduced.¹⁴⁹⁻¹⁵¹

Interfacing of TFA containing separations to MS is predominantly performed with analytical to capillary scale LC separations as conventional nano-ESI sources often lack robustness, even when run by qualified operators. Here we present a fast and robust nano-reversed phase-LC-MS method for IgG subclass-specific Fc N-glycopeptide profiling, using TFA containing mobile phases and a sheath-flow ESI sprayer for HPLC-MS interfacing. Fast separation is achieved on fused core particles at high flow rates. The sheath-flow ESI sprayer provides a plug-and-play zero dead volume interfacing and allows successful in-spray mixing of the LC gradient with TFA fix with minimal sample dilution. Moreover, MS contamination was found to be significantly reduced compared to standard nanoLC-MS interfacing. The robustness, sensitivity and reproducibility of the nanoLC-MS setup were evaluated with tryptic IgG digests. The method was applied to study pregnancy-associated IgG Fc glycosylation changes in a cohort of 26 women.

Experimental section

IgG purification and enzymatic cleavage. IgGs were affinity captured from total human serum or plasma and digested by trypsin as described previously.¹⁴⁸ Briefly, human polyclonal IgGs were captured from 2 µl plasma or serum by affinity chromatography with Protein A-SepharoseTM Fast Flow beads (GE Healthcare, Uppsala, Sweden), and cleaved overnight at 37°C with 200 ng sequencing grade trypsin (Promega, Madison, WI). The entire sample preparation was performed in 96-well plate format. Monoclonal antibody samples (provided by Hoffmann la Roche, Penzberg, Germany) were diluted in PBS and subjected to trypsin cleavage.

Fast nano-reversed phase-LC-ESI-MS. Separation was achieved on a Ultimate 3000 HPLC system (Dionex Corporation, Sunnyvale, CA), consisting of a degasser unit, binary loading pump, dual binary gradient pump, a temperature controlled autosampler maintained at 5°C and two column oven compartments maintained at 30°C. The Ultimate 3000 autosampler was fitted with a 10 µl PEEK sample loop. Samples were centrifuged at 4000 rpm for 5 min and 250-5000 nl were applied to a Dionex Acclaim PepMap100 C18 (5 mm x 300 µm i.d.) solid phase extraction (SPE) trap column conditioned with 0.1% TFA (Fluka, Steinheim, Germany; mobile phase A). In an alternative set up samples were applied to a 330 nl HALO C18 StemTrap SPE trap column (Optimize Technologies, Oregon City, USA) conditioned with mobile phase A. Sample loading was performed on the SPE trap column with mobile phase A at 25 µl/min for 1 min, followed by switching the SPE in-line with the gradient and separation column for 8 min. After 8 min the SPE was washed off-line by three full loop injections containing 5 µl 5% IPA + 0.1% FA and 5 µl 50% IPA + 0.1% FA. Separation was achieved on an Ascentis Express C18 nanoLC column (50 mm x 75 µm i.d., 2.7 µm HALO fused core particles; Supelco, Bellefonte, USA) conditioned at 900 nl/min with mobile phase A after which the following gradient of mobile phase A and 95% acetonitrile (ACN; Biosolve BV, Valkenswaard, the Netherlands; mobile phase B) was applied: 0 min 3% B, 2 min 5% B, 5 min 20% B, 6 min 30% B, 8 min 30% B, 9 min 0% B, and 14 min 0% B. The analytical column was protected for particulates coming from the sample injection by a stainless steel frit (2 µm pore size) mounted between the autosampler transfer tubing and the trap column. The HPLC system was coupled to a Dionex Ultimate UV detector (recording absorbance at 214 nm) equipped with a 3 nl flow cell. The HPLC was interfaced to a quadrupole-TOF-MS mass spectrometer (micrOTOF-Q; Bruker Daltonics, Bremen, Germany) with a standard ESI source (Bruker Daltonics) and a sheath-flow ESI sprayer (Figure 1; capillary electrophoresis ESI-MS sprayer; Agilent Technologies, Santa Clara, USA) applying the UV outlet tubing (20 µm i.d., 360 µm o.d.) as sprayer needle. To reduce the gas-phase TFA ion-pairing and assist with ESI spray formation a 2 µl/min sheath-flow of 50% IPA, 20% PA and 30% water was applied by one of the binary gradient pumps. To improve mobile phase evaporation a nitrogen stream was applied as dry gas at 4 l/min with a nebulizer pressure of 0.4 bars. Alternatively the LC-MS interfacing was achieved with a nano-ESI source (Bruker Daltonics) equipped with a metal nano-ESI needle (30 µm i.d.; Proxeon Biosystems, Odense M, Denmark). Scan spectra were recorded from 300 to 2000 dalton with 2 average scans at a frequency of 1 Hz. Quadrupole ion energy and collision energy of the MS were set at 2 and 4 eV, respectively. The total analysis time per sample was 16 min. The Ultimate 3000 HPLC system and the Bruker micrOTOF-Q were operated under Chromeleon Client version 6.8 and micrOTOF control version 2.3 software, respectively.

Data processing. Liquid chromatography-mass spectrometry datasets were calibrated internally using a list of known glycopeptides and were exported to the open mzXML format by Bruker DataAnalysis 4.0 in batch mode.¹⁵² Each dataset was then aligned to

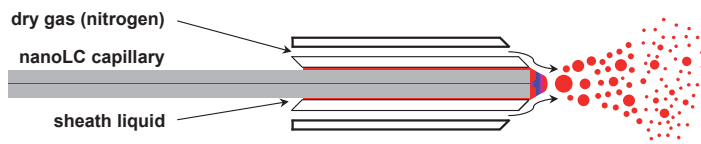


Figure 1. Schematic representation of the sheath-flow ESI sprayer.

a master dataset of a typical sample (containing many of the (glyco-)peptide species shared between multiple samples) using *msalign2*¹⁵³ and a simple warping script in AWK.¹⁵⁴ A list of 402 pre-defined features, defined as peak maximum (applied in this study) or peak area in specific mass and retention time windows, i.e. an accurate mass and time tag,¹⁵⁵ were then extracted from each dataset using the in-house developed software “Xtractor2D” and merged to a complete data matrix. As input, Xtractor2D takes one dataset in the mzXML format aligned to the master dataset and a reference list with pre-defined features with *m/z* windows and retention times in seconds. The retention times are on the chromatographic time scale of the master dataset used for the alignment and *m/z* values are calculated. The software and ancillary scripts are freely available at www.ms-utils.org/Xtractor2D. The complete sample-data matrix was finally evaluated using Microsoft Excel. Structural assignment of the detected glycoforms was performed on the basis of literature knowledge of IgG N-glycosylation.^{3,4,9,10,79,148} Relative intensities of the glycopeptide species (Table 1) derived from IgG1 (18 glycoforms), IgG4 (10 glycoforms), and IgG2 (18 glycoforms) were obtained by integrating and summing three isotopic peaks followed by normalization to the total IgG subclass-specific glycopeptide intensities.

On the basis of the normalized intensities the level of galactosylation, sialylation, bisecting N-acetylglucosamine, fucosylation, and the number of sialic acid moieties present on the galactose moieties (SA/Gal) were calculated according to the following formulas: Galactosylation = $(G1F + G1FN + G1FS + G1FNS + G1 + G1N + G1S) * 0.5 + G2F + G2FN + G2FS + G2FNS + G2 + G2N + G2S$ for IgG1 and IgG2, and $(G1F + G1FN + G1FS + G1FNS) * 0.5 + G2F + G2FN + G2FS + G2FNS$ for IgG4. Sialylation = $G1FS + G2FS + G1FNS + G2FNS + G1S + G2S$ for IgG1 and IgG2, and $G1FS + G2FS + G1FNS + G2FNS$ for IgG4. Bisecting GlcNAc = $G0FN + G1FN + G2FN + G1FNS + G2FNS + G0N + G1N + G2N$ for IgG1 and IgG2, and $G0FN + G1FN + G2FN + G1FNS + G2FNS$ for IgG4. Fucosylation IgG1 and IgG2 = $G0F + G1F + G2F + G0FN + G1FN + G2FN + G1FS + G2FS$. No fucosylation level was determined for the IgG4 subclass as the non-fucosylated species remained below the limit of detection. The number SA/Gal is calculated by dividing the prevalence of IgG sialylation by 2 * the level of galactosylation.

Glycopeptide profiling of a pregnancy cohort. Serum from 26 healthy pregnant Caucasian women without adverse obstetric history ranging in age between 24 and 40 years was obtained at three time points during pregnancy (1st, 2nd, 3rd trimester) and at three time points postpartum (6, 12, and 28-52 weeks).¹⁵ Human polyclonal

Table 1. Theoretical m/z values of human IgG Fc glycopeptides detected by nanoLC-ESI-MS.

Glycan species	IgG1 P01857 ^b		IgG2 P01859 ^b		IgG4 P01861 ^b	
	[M+2H] ²⁺	[M+3H] ³⁺	[M+2H] ²⁺	[M+3H] ³⁺	[M+2H] ²⁺	[M+3H] ³⁺
No glycan	595.260	397.176	579.265	386.513	587.262	391.844
G0F ^c	1317.527	878.687	1301.532	868.024	1309.529	873.356 ^{a1}
G1F	1398.553	932.705	1382.558	922.042	1390.556	927.373 ^{a2}
G2F	1479.580	986.722	1463.585	976.059	1471.582	981.391
G0FN	1419.067	946.380	1403.072	935.717	1411.069	941.049 ^{a3}
G1FN	1500.093	1000.398	1484.098	989.735	1492.096	995.066 ^{a4}
G2FN	1581.119	1054.416	1565.124	1043.752	1573.122	1049.084
G1FS	1544.101	1029.737	1528.106	1019.073	1536.104	1024.405 ^{a5}
G2FS	1625.127	1083.754	1609.132	1073.091	1617.130	1078.423
G1FNS	1645.641	1097.430	1629.646	1086.766	1637.643	1092.098
G2FNS	1726.667	1151.447	1710.672	1140.784	1718.670	1146.116
G0	1244.498	830.001	1228.503	819.338	-	-
G1	1325.524	884.019	1309.529	873.356 ^{a1}	-	-
G2	1406.551	938.036	1390.556	927.373 ^{a2}	-	-
G0N	1346.038	897.694	1330.043	887.031	-	-
G1N	1427.064	951.712	1411.069	941.049 ^{a3}	-	-
G2N	1508.090	1005.730	1492.096	995.066 ^{a4}	-	-
G1S	1471.072	981.051	1455.077	970.387	-	-
G2S	1552.098	1035.068	1536.104	1024.405 ^{a5}	-	-

^{a1} – ^{a5} isomeric glycopeptide species of IgG4 and IgG2.

^b SwissProt entry number

^c glycan structural features are given in terms of number of galactoses (G0, G1, G2), fucose (F), bisecting N-acetylglucosamine (N), and N-acetylneuraminic acid (S).

IgGs were purified with Protein G-Sepharose™ Fast Flow beads (GE Healthcare), digested overnight with trypsin and the resulting glycopeptides were analyzed by fast nano-reversed phase-LC-ESI-MS. Liquid chromatography-mass spectrometry datasets were processed automatically. Differences during pregnancy were evaluated using the Repeated Measures analysis (ANOVA) and differences between the 3rd pregnancy trimester and the last time point (28 to 52 weeks after delivery) were evaluated with the Paired-Samples T-test. *p*-Values were adjusted for multiple comparisons by Bonferroni correction, and *p*-values ≤ 0.013 were considered statistically significant. Data evaluation and statistical analysis were performed with Microsoft Excel and SPSS 16.0, respectively.

Results

Nano-reversed phase-LC-ESI-MS. In a first setup of the method, a tryptic IgG (glyco-)peptide pool prepared from plasma of a healthy donor by protein A affinity chromatography¹⁴⁸ was analyzed by direct injection of 250 nl onto the Ascentis Express C18 column. Separation was achieved at 500 nl/min using an ACN gradient with formic acid (FA) as acidic mobile phase additive to allow direct coupling with the mass spectrometer. The LC separation was interfaced to the mass spectrometer by a 30 μ m i.d. metal nanoLC-ESI-MS sprayer. Good separation of IgG Fc N-glycopeptides from different human IgG subclasses was obtained within a window of 15 min (Figure 2A). Sialylated IgG Fc N-glycopeptides of each specific subclass eluted later than the non-sialylated counterparts showing almost baseline separation.

To simplify LC-MS coupling and improve system robustness we interfaced the nanoLC separation to the mass spectrometer by a Agilent sheath-flow ESI sprayer (Figure 1) mounted in the standard ESI source at the default, fixed position. The UV outlet tubing (20 μ m i.d., 360 μ m o.d.) is applied directly as sprayer tip coupling the LC separation to the MS with zero dead volume. To assist with the electrospray formation a sheath-flow of 50% IPA in water with 2% PA was applied at 2 μ l/min. Furthermore, a nitrogen stream was applied as dry gas at 4 l/min with a nebulizer pressure of 0.4 bars to improve mobile phase evaporation.

The MS intensities of tryptic IgG Fc N-glycopeptides obtained with the sheath-flow sprayer (Figure 2B) were compared to those obtained with the metal nanoLC-ESI sprayer interface (Figure 2A). While IgG1 and IgG4 glycopeptides revealed similar intensities with both sprayers, the metal nanoLC-ESI sprayer provided higher IgG2 glycopeptide signals. In contrast to the metal nano-ESI sprayer the sheath-flow ESI sprayer provided a constant spray current which was independent of the LC gradient. This resulted in a more stable spray which performed equally well over the entire LC gradient. Moreover, the larger distance between the sheath-flow sprayer tip and the MS inlet resulted in a strong contamination reduction of the MS glass capillary as compared to the metal nanoLC-ESI sprayer.

To improve the separation and reduce the observed higher retention of sialylated glycopeptides FA was substituted for TFA. This resulted in sharper peaks and co-elution of sialylated IgG Fc N-glycopeptides with their non-sialylated counterparts (Figure 3). However, lower IgG glycopeptide signals were obtained (Figure 3A), which could be restored by increasing the PA concentration in the sheath-liquid to 20% (Figure 3B). Potential sample dilution and band broadening during ESI as a result of mixing with the sheath-liquid was evaluated by varying the sheath-flow rate from 1 to 10 μ l/min. Best results in terms of MS signal intensities were obtained at 2 μ l/min sheath-flow. Moreover, all sheath-flows revealed peaks of similar widths indicating that band broadening upon mixing is negligible. In addition to IPA, acetone, ACN and ethanol were evaluated as sheath-liquid additives at different flow rates but proved to be less effective than IPA in reducing/eliminating TFA gas-phase ion pairing.

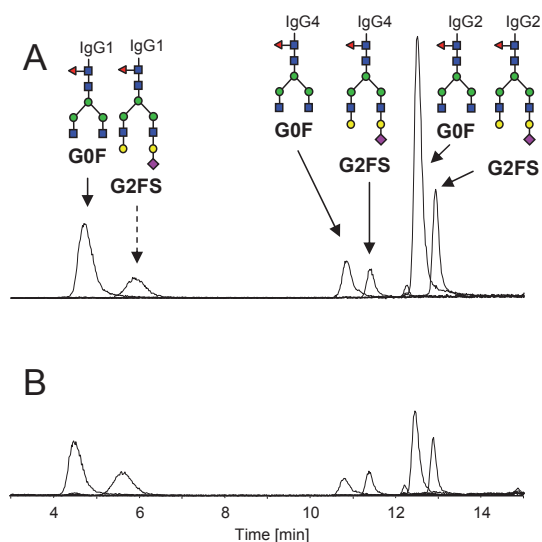


Figure 2. Comparison of the different sprayers for IgG Fc N-glycopeptide ionization. Extracted ion chromatograms obtained with a metal nano-ESI sprayer (A) and a sheath-flow ESI sprayer (B). Figures are represented with the same scaling. IgG Fc N-glycopeptides were separated at 500 nl/min using an ACN gradient with FA as mobile phase additive. The sheath-flow sprayer was applied with a 2 μ l/min sheath-flow of 50% IPA containing 2% PA. Glycan code as in Figure 1 of Chapter 1.

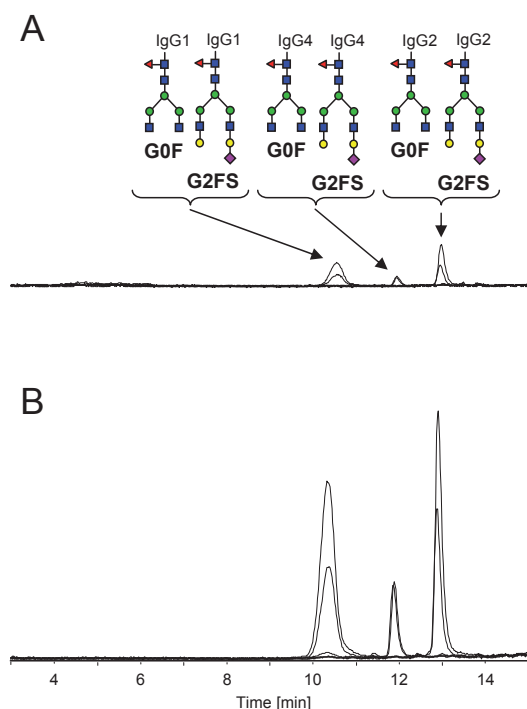


Figure 3. Effect of the PA concentration in the sheath-liquid on reduction of the TFA signal suppression. Extracted ion chromatograms of IgG glycopeptides with a 2 μ l/min sheath-flow of 50% IPA containing 2% (A) or 20% (B) PA. Figures are represented with the same scaling. IgG Fc N-glycopeptides were separated at 500 nl/min using an ACN gradient with TFA as mobile phase additive. Glycan code as in Figure 1 of Chapter 1.

To shorten the total analysis time (TAT) we increased the mobile phase flow rate to 900 nl/min and implemented a 330 nl HALO C18 StemTrap trap column for fast on-line analyte extraction and purification of large sample volumes. Extraction and separation were evaluated at three different temperatures (30, 40 and 60°C) showing low recoveries for all analytes with peak tailing (data not shown). Moreover, very broad IgG1 glycopeptide peaks were observed at 40°C, and peak splitting occurred at 60°C. In an effort to improve the analyte recoveries we replaced the StemTrap with a Dionex PepMap trap column and reevaluated the separations at the three different temperatures. While analyte recoveries were significantly improved, broad peaks were observed for separations at 40 and 60°C. Separation at 30°C provided sharp and symmetrical peaks for all subclass-specific IgG Fc glycopeptides. Moreover, separation could be achieved within 7 to 10 min using a short steep ACN gradient allowing a TAT of 16 min from sample to sample (Figure 4).

IgG Fc N-glycopeptides already eluted from the trap column within the first 8 min of the gradient. For efficient washing of the trap column and in order to minimize carryover, we switched the trap column off-line at 8 min and performed full loop injections (10 µl) onto the trap column with various IPA dilutions. With three full loop injections filling the loop with 5 µl 50% IPA containing 0.1% FA and 5 µl 5% IPA containing 0.1% FA the carryover was found to be below 1.5% throughout.

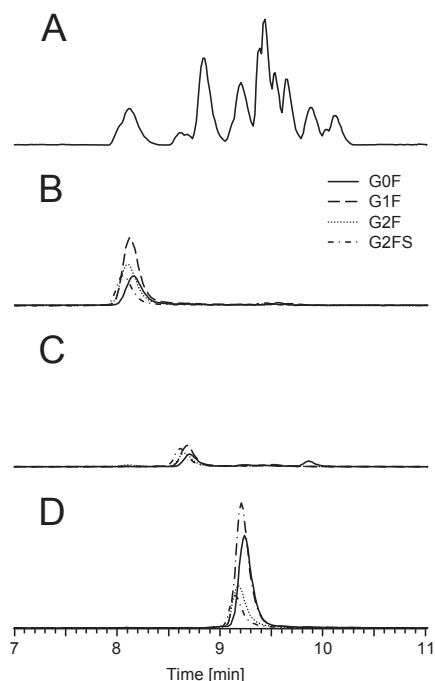


Figure 4. Fast nanoLC-ESI-MS of a tryptic IgG digest employing a sheath-flow sprayer. Base peak chromatogram of m/z 700-1700 (A); Extracted ion chromatogram of IgG1 (B), IgG4 (C), and IgG2 (D) Fc N-glycopeptides. Figures are represented with the same scaling. Separation was performed at 900 nl/min using an ACN gradient with TFA as mobile phase additive.

Optimization of mass spectrometry settings. In addition to the subclass-specific glycoforms (Table 1), using the standard (proteomics) MS settings IgG1, IgG4 and IgG2 glycopeptide decay products were observed as doubly charged fragment ions at m/z 1215.983, 1207.986 and 1199.988 Da, respectively. To minimize these decay products we evaluated various MS transfer parameters including capillary exit voltage, funnel voltages, quadrupole ion energy and collision energy. In the sheath-flow ESI setup, the capillary exit and funnel voltages hardly influenced the level of these decay products. By contrast, significant less decay products were observed when lowering the quadrupole ion energy (5 eV) and collision energy (10 eV) to 2 and 4 eV, respectively. While decreasing these energies resulted in lower total MS intensities, the signal to noise ratios remained unaffected. Tryptic IgG Fc N-glycopeptide ions were predominantly observed as $[M+3H]^{3+}$ and $[M+2H]^{2+}$ with low amounts of $[M+2H+Na]^{3+}$ (Figure 5). A total of 38 glycopeptide species derived from IgG1 (14 glycoforms), IgG4 (10 glycoforms), and IgG2 (9 glycoforms) were registered in both doubly charged and triply charged form for the healthy donor serum sample (Table 1) which is in accordance with previous reports on IgG Fc glycosylation profiling by LC-MS.^{4,79}

Repeatability. To evaluate the repeatability of the nanoLC-MS analysis a tryptic IgG Fc glycopeptide pool was analyzed eight times on three different days. Compound spectra were processed automatically which involved internal calibration, LC alignment and peak picking, revealing a good intra- and interday repeatability with lower than 5% relative standard deviation (RSD) of the normalized peak heights for the 8 major glycoforms of IgG1, IgG4 and IgG2 (data not shown). The robustness and repeatability of the entire analytical method including sample preparation (protein A affinity purification and tryptic cleavage) and mass spectrometric detection was assessed by performing three independent experiments comprising eight replicates each. The intraday variability for IgG1 (Figure 6A), IgG4 (Figure 6B), and IgG2 (Figure 6C) was determined within the three experiments and the interday repeatability was determined by comparing the results of the three experiments (Figure 6). For the 8 major IgG1, IgG4 and IgG2 glycoforms the RSDs for the intra- and interday repeatability were below 5% of the normalized peak heights, indicating that the variability of the sample preparation was below the variability of the nanoLC-MS analysis.

Limit of detection. To evaluate the limit of detection (LOD) of the reverse phase nanoLC-ESI-MS setup we prepared a dilution series with tryptic digests of two monoclonal antibodies ranging from 0.024 to 100 ng/ μ l and analyzed 1 μ l of each dilution. In the Fc portion of each antibody two N-glycans are located, which may vary in glycan composition. Upon analysis of the lowest measurable concentration three different glycoforms were observed. The LOD was estimated thereof to be 870 amol and was calculated by:

$$\frac{\text{Lowest measurable antibody concentration (0.195 ng}/\mu\text{l}) * \text{Number of Fc N-glycans (2)}}{\text{Molecular mass antibody (150 000) * Number of observed glycoforms (3)}}$$

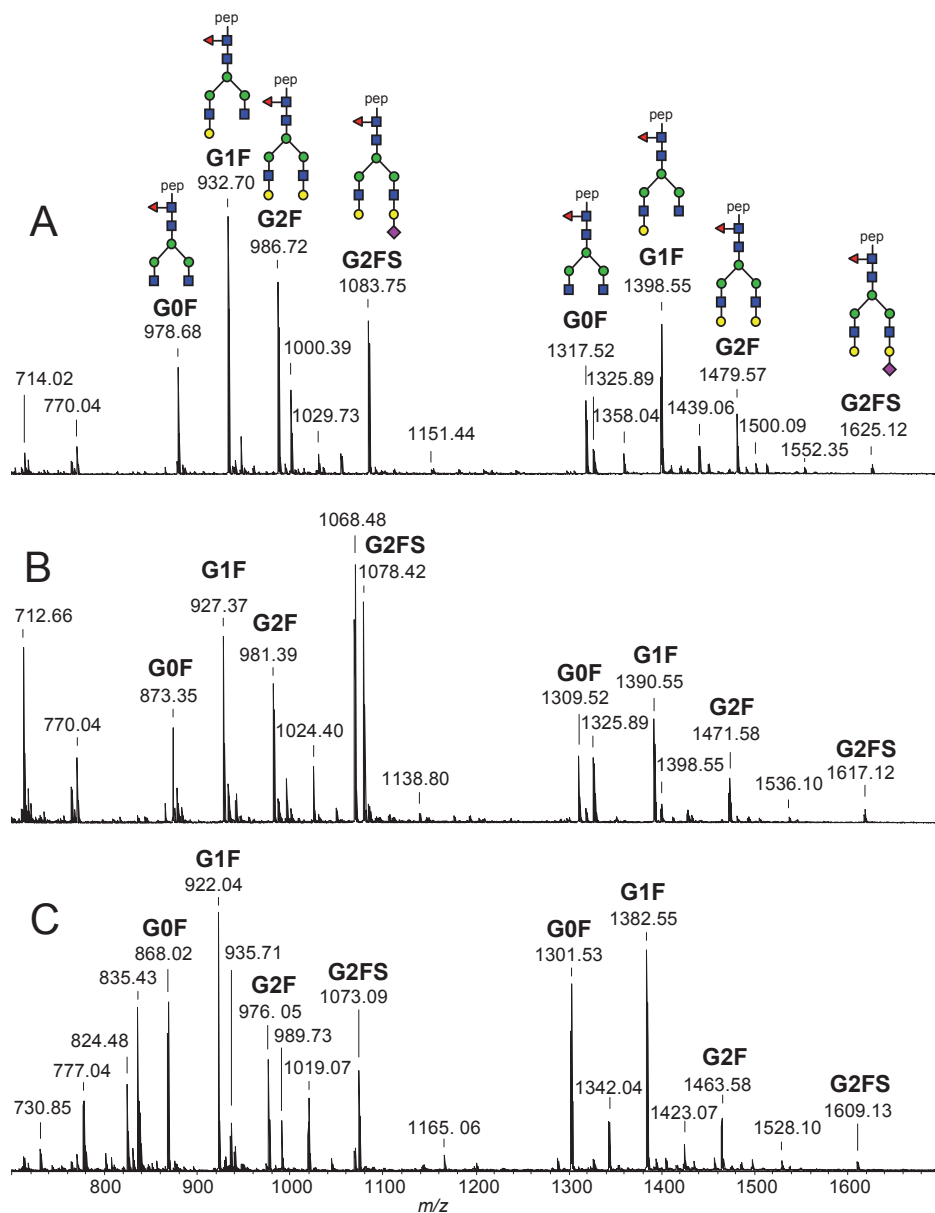


Figure 5. Tryptic glycopeptide mass spectra of IgG1 (A), IgG4 (B), and IgG2 (C) analyzed by fast nanoLC-ESI-MS with the sheath-flow ESI sprayer interface. Glycan code as in Figure 1 of Chapter 1.

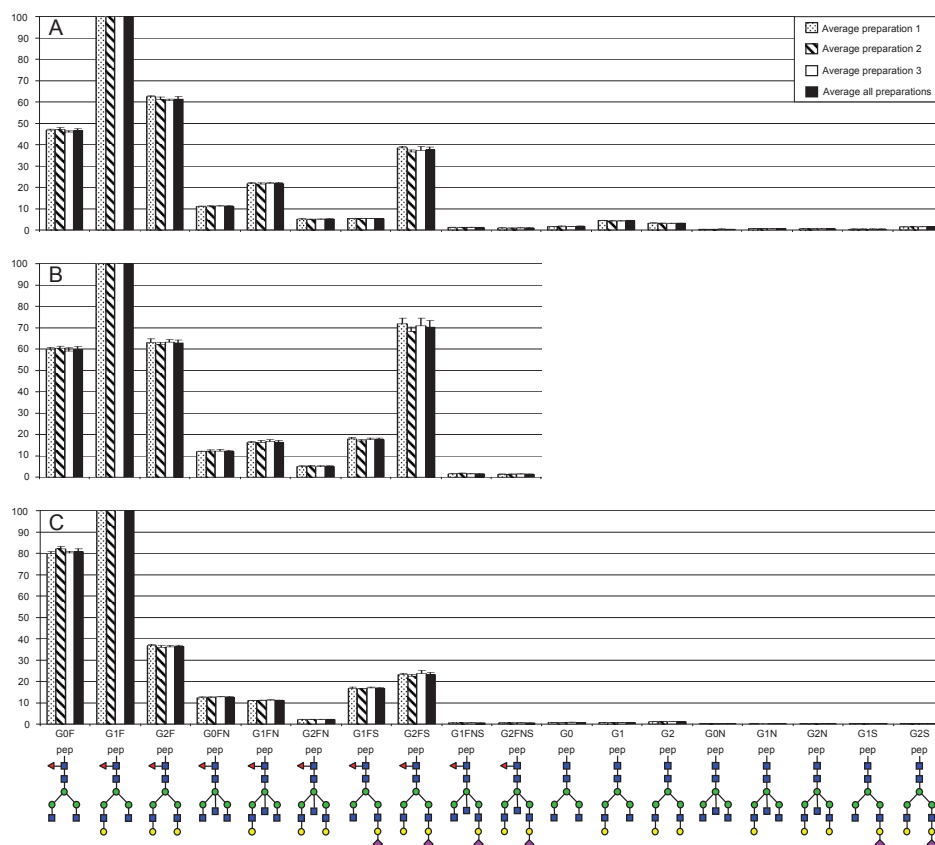


Figure 6. Repeatability of the sample preparation (protein A capturing, trypsin digestion) and analysis (fast nanoLC-ESI-MS with sheath-flow ESI sprayer interface) of IgG1 (A), IgG4 (B) and IgG2 (C). Relative intensities and standard deviations (STD) were determined from three independent experiments comprising eight replicates each. Glycan code as in Figure 1 of Chapter 1.

Pregnancy related IgG Fc N-glycosylation changes. The developed nano-reversed phase-LC-ESI-MS setup was applied to profile the IgG Fc N-glycosylation of 26 pregnant women at several time points during and after pregnancy. The obtained data revealed that the level of galactosylation, sialylation, and the number of SA/Gal increased significantly from the 1st to the 2nd pregnancy trimester for IgG1 (p -values < 0.005), IgG2/IgG3 (p -values < 0.010), and IgG4 (p -values < 0.015), while no significant increase was observed from 2nd to the 3rd pregnancy trimester (Table 2, Figure 7). During pregnancy no changes were observed for the level of fucosylation and bisecting N-acetylglucosamine of IgG1, IgG2/IgG3 and IgG4. The most prominent glycosylation changes between two consecutive time points were observed for the 3rd pregnancy trimester (time point 3) and 6 weeks *post partem* (time point 4; Table 2): all of the

analyzed glycosylation features, i.e., galactosylation, sialylation, SA/Gal, bisecting GlcNAc, and fucosylation showed significant changes (p -values < 0.001; Table 2). While galactosylation, sialylation, and SA/Gal were found to be decreased after delivery, the incidence of bisecting GlcNAc as well as fucosylation were increased for IgG1, IgG2/ IgG3, and IgG4 (Table 2, Figure 7).

In order to identify pregnancy-associated glycosylation changes which are supposed to be reversed 28-52 weeks after delivery, IgG glycosylation was compared between the 3rd pregnancy trimester (time point 3) and time point 6. Again, the levels of galactosylation, sialylation, and the number of SA/Gal for IgG1, IgG2/IgG3, and IgG4 were significantly higher in the 3rd pregnancy trimester compared to 28-52 weeks after pregnancy (p -values < 0.001). By contrast, significantly higher levels of bisecting GlcNAc and fucosylation were observed for IgG1 and IgG2/IgG3 28-52 weeks after pregnancy compared to the 3rd pregnancy trimester (p -values < 0.005).

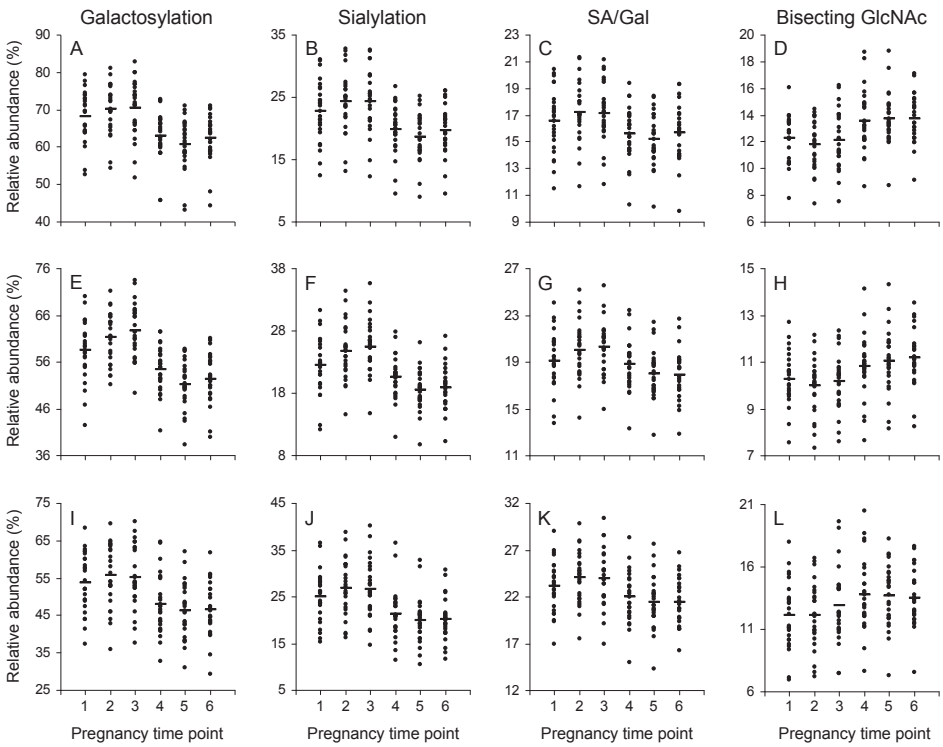


Figure 7. Pregnancy-associated IgG Fc N-glycosylation changes in 26 Caucasian women. For IgG1 (A-D), IgG2/IgG3 (E-H), and IgG4 (I-L) the level of galactosylation (A, E, I), sialylation (B, F, J), the incidence of SA/Gal (C, G, K) and the incidence of bisecting GlcNAc (D, H, L) are given for time point 1 (1st trimester), 2 (2nd trimester), 3 (3rd trimester), 4 (6 weeks after delivery), 5 (12 weeks after delivery), and 6 (28 to 52 weeks after delivery). Mean values are indicated by horizontal bars.

Table 2. Pregnancy related IgG Fc N-glycosylation changes.

Glycosylation feature	Time point	IgG1		IgG2/IgG3		IgG4	
		Mean (%)	<i>p</i> -value*	Mean (%)	<i>p</i> -value*	Mean (%)	<i>p</i> -value*
Galactosylation	1	68.34		59.02		53.24	
	2	70.16	< 0.001	61.35	0.009	55.06	0.012
	3	70.68	0.453	63.23	0.036	54.67	0.515
	4	63.13	< 0.001	54.68	< 0.001	47.55	< 0.001
	5	61.21	< 0.001	51.65	< 0.001	46.02	0.006
	6	61.92	0.140	52.07	0.338	46.44	0.469
Sialylation	1	22.98		22.76		24.72	
	2	24.22	< 0.001	24.55	< 0.001	26.46	< 0.001
	3	24.47	0.449	25.65	0.020	26.35	0.728
	4	19.83	< 0.001	20.61	< 0.001	21.08	< 0.001
	5	18.74	0.001	18.56	< 0.001	19.85	< 0.001
	6	19.44	0.023	18.73	0.544	19.96	0.768
SA/Gal	1	16.66		19.18		23.07	
	2	17.10	0.001	19.89	< 0.001	23.86	< 0.001
	3	17.14	0.734	20.18	0.019	23.92	0.578
	4	15.56	< 0.001	18.76	< 0.001	21.96	< 0.001
	5	15.16	0.017	17.86	< 0.001	21.37	< 0.001
	6	15.57	0.031	17.87	0.967	21.36	0.927
Bisecting GlcNAc	1	12.44		10.37		12.41	
	2	11.98	0.148	10.10	0.167	12.37	0.937
	3	12.35	0.232	10.28	0.329	13.22	0.108
	4	13.79	< 0.001	10.98	< 0.001	14.03	< 0.001
	5	13.81	0.921	11.16	0.092	13.91	0.605
	6	13.80	0.956	11.21	0.534	13.54	0.041
Fucosylation	1	92.24		97.28		n.d.	n.d.
	2	92.24	0.966	97.19	0.134	n.d.	n.d.
	3	92.03	0.362	97.00	0.003	n.d.	n.d.
	4	92.90	< 0.001	97.34	< 0.001	n.d.	n.d.
	5	93.15	0.071	97.42	0.253	n.d.	n.d.
	6	92.86	0.111	97.40	0.816	n.d.	n.d.

*Repeated Measures analysis *p*-values < 0.013 are considered to be significant and are highlighted in bold. *p*-Values represent differences between time point 1 vs. 2, 2 vs. 3, 3 vs. 4, 4 vs. 5 and 5 vs. 6. n.d., not determined.

Discussion

Here we describe a fast nanoLC-MS method for detailed Fc N-glycosylation profiling of human IgG subclasses in a single analysis. Human polyclonal IgGs were purified from plasma by protein A (captures IgG1, IgG2, and IgG4) or protein G (captures IgG1-4) affinity chromatography and digested overnight with trypsin in 96-well plate format. Aliquots of the samples containing IgG (glyco)peptides were loaded onto a C18 trap column and separated on a fused core C18 nanoLC column. In a first version of the setup, samples were injected directly onto the nanoLC column. The LC was interfaced to the MS by a metal nano-ESI sprayer and separation was performed with a gradient of ACN (mobile phase B) and FA (mobile phase A). No acid was added to the ACN to avoid potential acid-catalyzed hydrolysis of ACN to acetic acid and ammonia¹⁵⁶⁻¹⁵⁸ which might result in the formation of ammonium adducts. The metal nano-ESI sprayer was set close to the glass capillary inlet and the position was optimized manually to obtain the best ESI current.

During the LC gradient the increase in the ACN content of the mobile phase resulted in an increase of the total ion current and higher MS intensities later in the gradient. On reverse phase LC columns IgG glycopeptides elute at a relative low percentage of ACN and thus experience less favorable ESI spray conditions with standard nano-ESI sources. By replacing the nano-ESI source and sprayer with a conventional ESI source and a sheath-flow ESI sprayer we obtained a plug-and-play zero dead volume nanoLC-MS interface. Compared with regular nano-ESI sprayers, the sheath-flow ESI sprayer is positioned considerably further away from the glass capillary inlet. This reduced contamination of the MS and allowed many sequential injections without intensity loss. The volatile sheath-liquid required for proper spraying with the sheath-flow sprayer provided a very constant spray and ionization over the entire gradient with MS intensities early in the gradient being similar to those observed with a metal nano-ESI sprayer. The sheath-liquid composition was optimized to enhance analyte signal intensities,¹⁵⁹ and reduce TFA gas-phase ion-pairing with basic analytes by introducing TFA fix^{149,151} during spray formation without excessive sample dilution. We expect that the sheath-flow ESI sprayer will find acceptance for other applications which require robust and sensitive coupling of nanoLC to mass spectrometry.

With standard porous particle nanoLC columns separation is achieved with mobile phase flow rates between 100-300 nl/min generating pressures up to 200 bars. Current fused core particle columns offer higher efficiency due to the combination of a smaller longitudinal diffusion coefficient and lower eddy diffusion¹⁶⁰ and can be applied at higher mobile phase flow rates resulting in efficient separation without requiring ultra-high pressures.¹⁶¹ Moreover, similar resolving power (plate height) has been reported for both low and high mobile phase flow rates. We, therefore, increased the mobile phase flow rate 1.8 times which effectively reduced the TAT to 16 min. Separation at 30°C of StemTrap extracted analytes showed peak tailing for all IgG Fc N-glycopeptides which could not be eliminated by diluting the sample or lowering the injection volume. Tailing

with HALO stationary phases has been reported for separations of basic peptides at lower temperatures which disappeared when the temperature was increased to 60°C.¹⁶² We, therefore, explored this possibility by increasing the separation temperature up to 60°C. While recoveries were comparable at the three temperatures, tailing of the analyte peaks increased at higher temperatures with even peak splitting at 60°C, which suggests that the tailing was a result of the relatively low loading capacity of the fused core trap column (surface area ~150 m²/g). To improve analyte recoveries and eliminate peak tailing we, therefore, replaced the fused core trap column by a porous particle trap column (surface area ~300 m²/g). Both porous particle trap column and fused core particle analytical column revealed similar retention for the IgG Fc N-glycopeptides providing a system with high capacity sample extraction and fast analyte separation.

The soft nature of ESI allows analysis of intact glycopeptides including sialylated glycopeptides. However, at standard (proteomics) MS settings glycopeptides are prone to show in-source decay and/or fragmentation during ion-transfer.^{137,139} We observed doubly charged IgG glycopeptide ions which only occur when the linkage between one of the antenna GlcNAc residues and the core mannose breaks. By lowering the default collision energy and quadrupole ion energy occurrence of these decay products could almost be reduced to noise levels. The funnel setup of the quadrupole-TOF-MS did not appear to influence the level of glycopeptide decay products observed, indicating a softer ion transfer compared to the setup with skimmer and lenses used in the TOF-MS (Bruker micrOTOF™) where lowering the voltage difference between the capillary exit and lens 1 was required to reduce these decay products (data not shown).

Simultaneous glycosylation profiling of different IgG subclasses can be achieved by direct infusion-MS,¹³⁷ MALDI-MS^{102,148} and LC-MS.^{4,79} Detailed glycosylation analysis of human polyclonal IgG by direct infusion-MS and MALDI-MS is limited by the presence of isomeric glycopeptide species.¹⁴⁸ By contrast, LC-MS provides detailed glycosylation profiles of different IgG subclasses in a single analysis.^{4,79} The nanoLC-MS method described in this manuscript allows fast and detailed subclass specific analysis of IgG Fc N-glycosylation with a similar LOD but a substantially shorter TAT (16 min) compared to previously described methods.^{4,79} Due to the sheath-flow ESI sprayer interface the system remains highly stable over a long period of time (more than 3 weeks) without the requirement of MS cleaning and maintenance (data not shown). The ionic interactions of sialylated glycopeptides with residual silanol groups on the silica based RP stationary phase is suppressed by the presence of TFA improving chromatographic resolution.¹²⁹ Due to the predominantly peptide-based retention the complete IgG subclass specific glycopeptide profile is obtained in a 30 s separation window (Figure 4). The here developed robust, fast and sensitive method for IgG Fc glycosylation analysis will not only be suitable to analyze the glycosylation of clinical IgG samples but will also be applicable in a biotechnological setting for the characterization of recombinantly produced therapeutic IgGs.

For a variety of diseases lowered IgG galactosylation has been reported.¹⁴⁷ In healthy individuals IgG Fc N-glycosylation is known to be influenced by age, gender and pregnancy.^{4,8-10,13,14} During pregnancy a decreased percentage of agalactosylated IgG has been reported which after delivery increased to values similar to before conception.¹⁴ The here developed fast nano-reversed phase HPLC-MS method was successfully applied to profile IgG Fc N-glycosylation of 26 pregnant women, confirming the previous reported increase of IgG galactosylation during pregnancy.¹⁵ The applied technique additionally allowed sensitive and accurate determination of sialylated IgG glycopeptides, revealing increased levels of IgG1, IgG2/IgG3, and IgG4 sialylation during pregnancy. The pregnancy-associated increase in sialylation was found to be more pronounced than the increase in galactosylation. This is reflected by the fact that the ratio of SA/Gal is higher during pregnancy compared to after delivery (Figure 7C, G, and K). This finding indicates that IgG Fc galactosylation and sialylation, although they tend to show some correlation,¹⁵ are not fully concerted in terms of regulation.

Furthermore, we observed lower levels of IgG1 and IgG2/IgG3 bisecting GlcNAc and fucosylation during pregnancy than after delivery. Differences in IgG glycosylation are postulated to be a reflection of altered N-glycan biosynthesis in B-lymphocytes.^{12,57} Thus the decreasing levels of bisecting GlcNAc and fucosylation with increasing levels of galactosylation and sialylation during pregnancy may indicate altered activities of GlcNAc transferase III (catalyzes the addition of a β -1,4-linked GlcNAc to the β -linked mannose of the trimannosyl core),¹⁶³ α 1,6-fucosyltransferase (catalyzes the addition of a fucose residue to the innermost GlcNAc of the chitobiose core),¹⁶⁴ β 4-galactosyltransferase(s) (catalyzes the addition of β 1,4-linked galactoses to the GlcNAc residues),¹⁶⁵ and α 2,6/ α 2,3-sialyltransferase (catalyzes the addition of α 2,6 or α 2,3-linked sialic acids to the antennae galactose residues).^{166,167} Future studies will hopefully elucidate the regulatory mechanisms involved in inducing pregnancy-associated glycosylation changes as well as the immunological role and implications of these alterations.

In conclusion, the here described nano-reversed phase HPLC-MS method allows sensitive, fast and reproducible analysis of subclass-specific IgG glycosylation and is combined with automated data processing. Moreover, the sheath-flow ESI sprayer provides a sensitive and robust plug-and-play interface that minimizes contamination of the mass spectrometer. The system is currently being applied to analyze large clinical cohorts, and no decrease in signal intensity has been observed after 2000 injections.

Acknowledgements

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Cotton HILIC SPE microtips for micro-scale purification and enrichment of glycans and glycopeptides

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Abstract

Solid phase extraction microtips are important devices in modern bioanalytics, as they allow miniaturized sample preparation for mass spectrometric analysis. Here we introduce the use of cotton wool for the preparation of filter-free HILIC SPE microtips. To this end, pieces of cotton wool pads (approximately 500 μg) were packed into 10 μl pipette tips. The performance of the tips was evaluated for micro-scale purification of tryptic IgG Fc N-glycopeptides. Cotton wool HILIC SPE microtips allowed the removal of salts, most non-glycosylated peptides, and detergents such as SDS from glycoconjugate samples. MALDI-TOF-MS glycopeptide profiles were very repeatable with different pristine tips as well as reused tips, and very similar profiles were obtained with different brands of cotton wool pads. In addition, we used cotton HILIC microtips to purify N-glycans after PNGase-F treatment of IgG and transferrin followed by MALDI-TOF-MS detection. In conclusion, we establish cotton wool microtips for glycan and glycopeptide purification with subsequent mass spectrometric detection.

Introduction

Due to its speed, resolution and sensitivity, modern mass spectrometry provides great opportunities for detailed structural characterization of protein glycosylation including protein identification, determination of site-specific glycosylation profiles, and structural characterization of glycans at the level of glycopeptides and released glycans. For the efficient ionization and detection of glycopeptides and glycans, enrichment or purification steps are often required. Next to graphitized carbon solid phase extraction (SPE), hydrazine coupling, and lectin or antibody affinity chromatography, hydrophilic interaction liquid chromatography (HILIC) has been found to be very suitable for this purpose.¹⁶⁸⁻¹⁷⁰ While the specificity of lectins and antibodies often only allows the isolation of a subset of glycans or glycopeptides from a pool, HILIC and graphitized carbon SPE can be employed for isolation of a broad range of glycoconjugates, making these adsorption chromatography methods applicable in a wide range of glycomics and glycoproteomics studies.¹⁶⁸⁻¹⁷⁰

HILIC SPE has been found to be particularly useful for the enrichment of tryptic N-glycopeptides. For this purpose, ZIC-HILIC stationary phases have been applied in ion-pairing mode using trifluoroacetic acid as a mobile phase additive.¹³⁸ Alternatively, carbohydrate-based stationary phases such as Sepharose and microcrystalline cellulose have been used for N-glycopeptide isolation.^{102,148} An important feature of these carbohydrate-based stationary phases is that they are non-ionic, and HILIC adsorption is, therefore, dominated by hydrogen bonding of the glycan moieties with the stationary phase, while non-glycosylated peptides, lipids, salts and detergents tend to show low retention.^{94,138,139} Retention of glycoconjugates is usually achieved with acetonitrile concentrations in the range of 80% while elution is performed with high water content.¹³⁹ The required elution conditions make HILIC well compatible with mass spectrometry, both in online and off-line mode.^{92,138,139,171}

Immunoglobulin G (IgG) Fc N-glycosylation profiles can be analyzed in a reproducible and robust manner by MALDI-MS after HILIC SPE with Sepharose as well as microcrystalline cellulose performed in batch mode or at the 96-well plate format.^{102,148} Here we introduce the use of cotton wool in microtips as versatile HILIC micro-SPE tool and demonstrate the reproducible application of these tips for isolation of tryptic IgG Fc N-glycopeptides from human plasma, followed by subclass-specific glycosylation profiling at the glycopeptide level. Moreover, the cotton wool microtips were successfully applied for the purification and enrichment of released N-glycans from detergent-containing glycoprotein samples treated with PNGase-F.

Experimental section

IgG purification. Polyclonal human IgGs were purified from plasma by affinity chromatography with immobilized protein A as described previously,⁴ with minor

modifications. Briefly, rProtein A-Sepharose™ beads (GE Healthcare, Eindhoven, The Netherlands) were washed three times with PBS. To each well of a 96-well OF1100 filter plate (Orochem Technologies Inc., Lombard, IL) 50 µl PBS, 50 µl of slurry containing ca. 5 µl of beads and 2 µl of human plasma (containing ca. 20 µg of IgG) were applied. The plate was covered with a lid and incubated at room temperature with gentle agitation for 1 h. After incubation, beads were washed with 3x 200 µl PBS and 3x 200 µl of water on a vacuum manifold. Captured human polyclonal IgGs were released from protein A by 5 minutes incubation with 40 µl of 100 mM formic acid (Fluka, Steinheim, Germany), eluted into a polypropylene 96-well V-bottom plate (V96 microwell; NUNC, Roskilde, Denmark) by centrifugation (1 min at 18 g). After centrifugation, the eluates were neutralized (final pH > 7) with 20 µl of 300 mM ammonium bicarbonate. Alternatively, the eluates were dried by vacuum centrifugation for 2 hours.

Trypsin digestion. Tosyl phenylalanyl chloromethyl ketone (TPCK) treated trypsin (Sigma-Aldrich, Steinheim, Germany) was dissolved in ice-cold 20 mM acetic acid (Merck, Darmstadt, Germany) to a final concentration of 0.05 µg trypsin per µl and stored in aliquots at -80°C until use. To each of the neutralized IgG samples 8 µl of the trypsin stock (400 ng in total) and 12 µl of water were added. The samples were shaken for 3 min and incubated overnight at 37°C. Tryptic IgG digests were stored at -20°C until Sepharose or cotton HILIC desalting and purification. Bovine fetuin (1 mg; Sigma-Aldrich) was dissolved in 200 µl 50 mM ammonium bicarbonate containing 10 mM DTT and reduced at 60°C for 40 min. Cysteine alkylation was achieved by addition of 30 µl 100 mM iodoacetamide dissolved in 50 mM ammonium bicarbonate followed by 30 min incubation at room temperature in the dark. The alkylation reaction was stopped by putting the sample under a fluorescent lamp (gas discharge lamp) for 30 min. Trypsin digestion was achieved overnight at 37°C with 20 µg sequencing grade modified trypsin (Promega, Madison, WI).

N-glycan release. N-glycans from protein A purified IgGs were released as described previously.⁹⁴ Briefly, the dried IgG samples were denatured with 2 µl SDS (2%) at 60°C for 10 min. Subsequently, 2 µl of a release mixture containing 2% NP-40, 2.5x PBS and 0.5 mU PNGase-F (Roche, Mannheim, Germany) was applied. The samples were incubated overnight at 37°C for N-glycan release. Human apo-transferrin (0.1 mg; Sigma-Aldrich) was dissolved in 21 µl 50 mM ammonium bicarbonate containing 10 mM DTT and reduced at 60°C for 40 min. Cysteine alkylation was achieved by adding 4 µl 200 mM iodoacetamide containing 50 mM ammonium bicarbonate followed by 30 min incubation at room temperature in the dark. The alkylation was stopped by putting the sample under a fluorescent lamp (gas discharge lamp) for 30 min. To the sample 6 mU PNGase-F was applied and incubated overnight at 37 °C for N-glycan release.

Sepharose HILIC SPE in 96-well plate format. Sepharose (45-165 µm; GE Healthcare, Uppsala, Sweden) HILIC SPE was performed as described previously.¹⁴⁸

Preparing cotton HILIC SPE microtips. Cotton wool pads of three different brands (Da, Dynaretail, Leusden, The Netherlands; Etos, Etos bv, Beverwijk, the Netherlands; Bella, Groupe Lemoine; Paris, France) were purchased in local stores and used for the preparation of HILIC SPE microtips. According to the manufacturers the cotton wool pads consist of 100% pure cotton. A small piece of cotton wool with a weight of approximately 500 µg was taken from a cotton wool pad and pushed into the end part of a 10 µl pipette tip (Rainin, Tiel, The Netherlands) using a blunt needle. Microtips were stored in a closed box until use.

Cotton HILIC micro-SPE of tryptic IgG glycopeptides and N-glycans. The cotton HILIC SPE microtip was washed with 5 times 10 µl of water and conditioned with 3 times 83% of acetonitrile (Biosolve BV, Valkenswaard, The Netherlands) by aspirating and dispensing the solution. For less than 10% of the prepared tips the flow upon solvent aspiration was found to be slow and insufficient, and such tips were therefore discarded. For sample application to the cotton HILIC SPE microtip, 39 µl of acetonitrile was added to 8 µl of a tryptic IgG digest (corresponding to ca. 2 µg of IgG), a fetuin digest or an N-glycan release sample, and the mixture was pipetted up and down 20 times to allow glycoconjugate adsorption. The adsorbed glycoconjugates were washed 3 times with 10 µl of 83% acetonitrile containing 0.1% TFA and eluted directly onto a MALDI plate with 2 µl water.

Capacity and recovery of cotton HILIC micro-SPE. To evaluate the capacity and recovery of cotton HILIC micro-SPE, maltoheptaose (Sigma-Aldrich) was labeled with 2-aminobenzoic acid (2-AA; Sigma-Aldrich) and analyzed by HILIC-HPLC with fluorescence detection (HPLC-Flu) as described previously.⁹⁴ The labeled maltoheptaose stock was used to prepare a standard dilution series ranging from 0.98-1000 µg/ml. From each standard dilution 10 µl was diluted with 90 µl acetonitrile and applied to the cotton HILIC SPE microtip. The adsorbed maltoheptaose was washed 3 times with 10 µl of 90% acetonitrile containing 0.1% TFA and eluted into a 96-well storage plate with 10 µl water. The standard dilutions and cotton micro-SPE preparations were diluted 10 times with water, brought to 75% acetonitrile, and analyzed by HPLC-Flu (injection of 20 µl) in the order of increasing concentrations.

MALDI-TOF-MS. For IgG Fc N-glycopeptide profiling by reflectron positive mode MALDI-TOF-MS 2 µl of the Sepharose purified glycopeptides or glycopeptides eluted directly from cotton HILIC SPE microtips with 2 µl of water were spotted onto a polished stainless steel MALDI plate (Bruker Daltonics, Bremen, Germany) and allowed to air dry. Samples were overlaid with 2 µl α-cyano-4-hydroxycinnamic acid (CHCA, 5 mg/ml in 50% acetonitrile; Bruker Daltonics) and allowed to air dry. For glycosylation profiling in the linear positive mode, IgG glycopeptides were directly eluted onto an AnchorChip 600/384 MALDI plate (Bruker Daltonics) and allowed to air dry. Samples were overlaid with 1 µl dihydroxybenzoic acid (DHB, 5 mg/ml

in 50% acetonitrile with 0.1% TFA; Bruker Daltonics). The AnchorChip plate was covered with a pierced cap containing 5 holes of ca. 5 mm diameter, allowing the DHB matrix to air dry at room temperature in a controlled manner. Samples were analyzed on an Ultraflex II MALDI-TOF/TOF-MS (Bruker Daltonics), and mass spectra were processed with flexAnalysis software (Bruker Daltonics) as described previously.¹⁴⁸ Similarly, N-glycans were eluted from cotton HILIC SPE microtips, spotted directly onto an AnchorChip MALDI plate, allowed to air dry, and were overlaid with 1 µl DHB.

nanoLC-ESI-IT-MS(/MS) of fetuin. Tryptic fetuin digests (250 nl) were analyzed as described previously, with minor modifications.¹⁷² Briefly, separation was achieved on a Ultimate 3000 nanoLC system (Dionex, Amsterdam, The Netherlands) equipped with a reverse phase guard (C18 PepMap, 300 µm × 5 mm; Dionex) and analytical column (C18 PepMap, 3 µm; 75 µm × 100 mm; Dionex). The column was equilibrated at room temperature with eluent A (0.1% formic acid in water) at a flow rate of 300 nl/min. After injection of the samples, a gradient was applied to 25% eluent B (95% acetonitrile, 5% water) in 15 min and 70% eluent B at 25 min followed by an isocratic elution with 70% eluent B for 5 min. The separation was monitored by UV absorption at 215 nm. The LC system was interfaced to an Esquire HCTultra ESI-IT-MS (Bruker Daltonics, Bremen, Germany) by a metal nano-ESI sprayer (Proxeon Biosystems, Odense, Denmark). The MS was operated in the positive-ion mode at 1250 V. The solvent was evaporated at 170°C with a nitrogen stream of 6 l/min. Ions from *m/z* 600-1600 were registered. Automatic fragment ion analysis was enabled, resulting in MS/MS spectra of the most abundant peaks.

Results

IgG purification and tryptic cleavage. IgGs were affinity-captured from 2 µl of human plasma (containing ca. 20 µg IgG) in 96-well filter plates containing 5 µl of Protein A-Sepharose beads, followed by the elution of IgGs with 40 µl of 100 mM formic acid. While a previously described version of the protocol involves drying of the sample by vacuum centrifugation,^{4,148} this procedure was replaced in the current protocol by a neutralization step with ammonium bicarbonate. Tryptic cleavage of IgGs was performed by an overnight incubation at 37°C either with 200 ng sequencing grade modified trypsin or 400 ng TPCK-treated trypsin. IgG glycopeptides were purified by Sepharose HILIC SPE in 96-well plate format¹⁴⁸ and analyzed by MALDI-TOF-MS in the reflectron positive mode. The IgG Fc N-glycopeptide profiles obtained for the neutralization procedure were very similar to those observed with the previously described vacuum centrifugation procedure¹⁴⁸ independent of the trypsin used (data not shown).

Glycopeptide purification using cotton HILIC microtips. Cotton was evaluated for its potential as a stationary phase in HILIC SPE of IgG glycopeptides. To this end, a small piece of a cotton wool pad (approximately 500 μg) was packed into the end of a pipette tip (Figure 1). MALDI-TOF-MS profiles obtained from blank elutions using cotton HILIC SPE microtips were evaluated and found to be virtually identical to matrix controls (only matrix spotted). Moreover, no cotton wool-related contaminant peaks were detected in the MALDI-TOF-MS profiles (data not shown).

Next, the obtained SPE microtips were tested for HILIC mode enrichment of IgG glycopeptides. Specifically, acetonitrile was added to an aliquot (8 μl) of a tryptic digest of human plasma IgG (corresponding to ca. 2 μg intact IgG), and glycopeptides were adsorbed to the HILIC micro-SPE stationary phase. After three washes with 10 μl of 83% acetonitrile containing 0.1% TFA, the retained glycopeptides were eluted directly onto a MALDI plate with 2 μl of water, followed by MALDI-TOF-MS profiling of IgG Fc N-glycopeptides. The obtained MALDI profiles represent the N-glycopeptide microheterogeneity of IgG1, 2 and 4 subclasses. Examples of the glycopeptide profiles registered by reflectron mode and linear mode MALDI-TOF-MS are shown in Figure 2. Structural assignment of the detected glycoforms was performed on the basis of

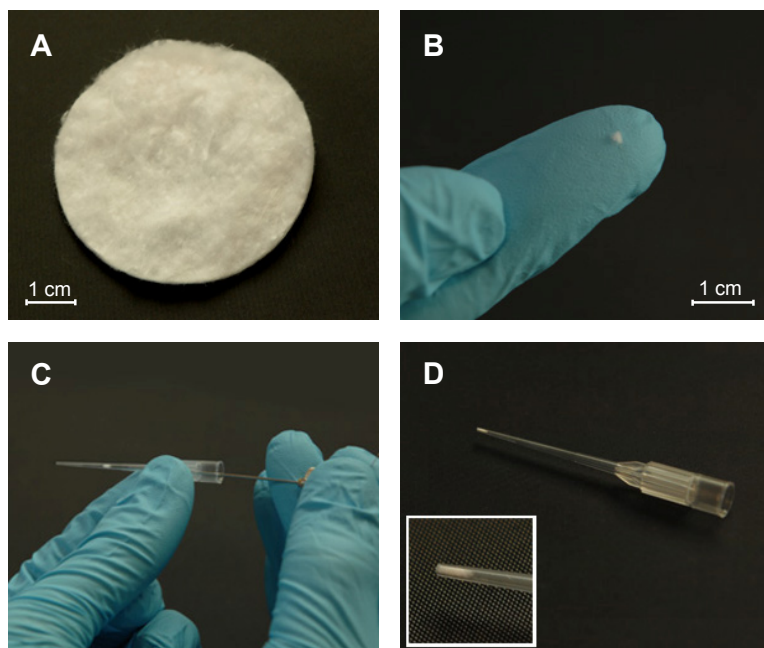


Figure 1. Preparation of a cotton HILIC SPE microtip. From a cotton wool pad (A), approximately 500 μg is taken (B) and pushed into a 10 μl pipette tip using a blunt metal needle (C). The cotton is pushed down into the end part of the tip (D).

literature knowledge of IgG N-glycosylation.^{3,4,9,10,79,148} The most abundant signals were obtained for the glycopeptides of IgG2 followed by IgG1 and IgG4. While reflectron mode MALDI-TOF-MS analysis provided IgG glycopeptide spectra with isotopic resolution, only neutral glycoforms were observed. By contrast, linear mode MALDI-TOF-MS allowed low resolution detection of both sialylated glycopeptides and species with neutral glycoforms.

In addition to cotton we evaluated 1 and 3 mm Whatman cellulose chromatography paper for IgG glycopeptide enrichment. Glycopeptide profiles were obtained which were similar to those observed with cotton (data not shown). However, due to complications with inserting small pieces of paper into microtips without blocking the tip this approach was stopped.

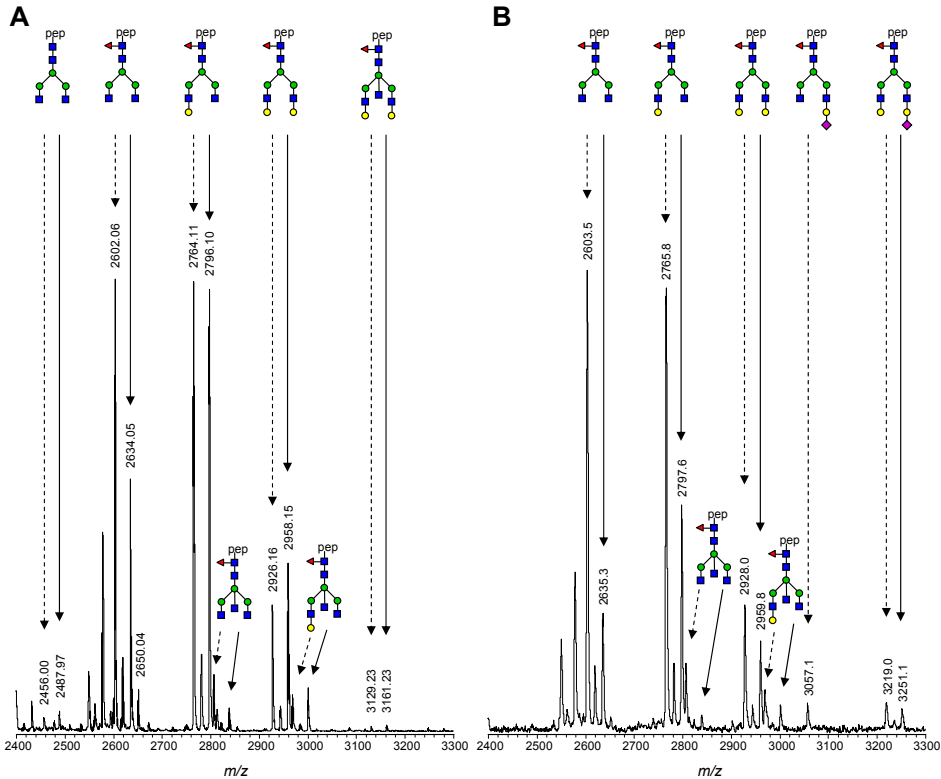


Figure 2. MALDI-TOF-mass spectrometric profiles of IgG glycopeptides prepared using cotton HILIC SPE microtips. Mass spectra were registered in reflectron positive mode using CHCA matrix (A) and in linear positive mode using DHB matrix (B). Continued arrows, IgG1 glycopeptides; dashed arrows, IgG2 glycopeptides; glycan code as in Figure 1 of Chapter 1.

Validation of cotton HILIC micro-SPE. One cotton HILIC microtip was used 8 times for glycopeptide purification from a tryptic IgG digest pool followed by reflectron mode MALDI-TOF-MS of the eluted glycopeptides (Figure S-1 A and B). This experiment was repeated on four different days using each time a new cotton HILIC SPE tip. In another set of experiments, 8 different tips were used for IgG Fc N-glycosylation profiling from the above-mentioned tryptic IgG digest pool (Figure S-1 C and D). This experiment was also repeated on four different days using new tips for each experiment. Highly reproducible IgG Fc N-glycosylation profiles were obtained after cotton HILIC micro-SPE, independent of using the same tip repeatedly or using different tips for micro-scale purification.

Validation of the complete method. IgG Fc N-glycosylation profiling was performed in parallel on eight plasma aliquots of a control individual. This involved Protein A capturing, neutralization of the eluate, cleavage using TPCK-treated trypsin, cotton

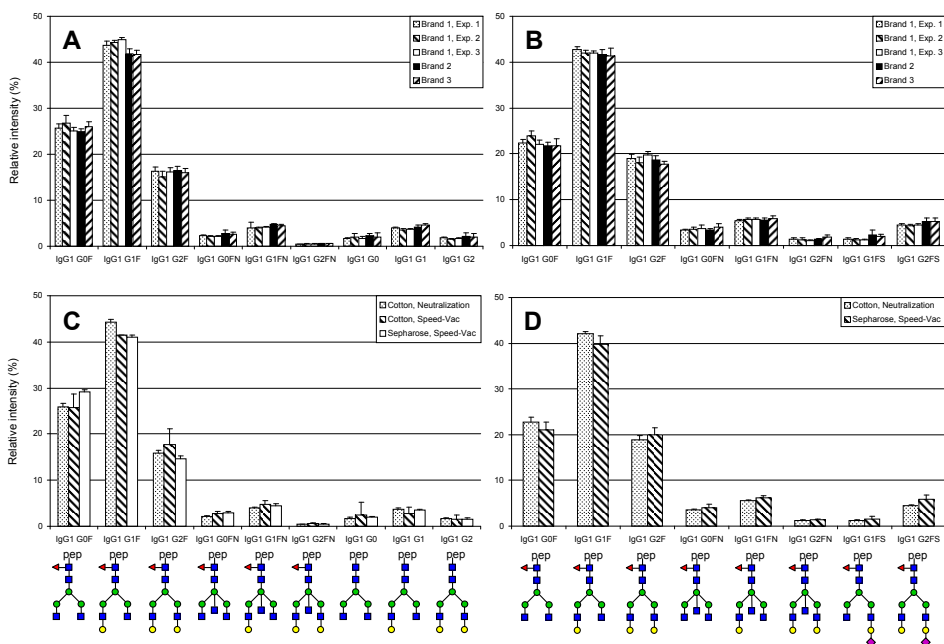


Figure 3. Repeatability of IgG1 glycopeptide profiling applying cotton HILIC SPE microtips. IgG1 glycopeptides were detected by MALDI-TOF-MS in reflectron positive mode using CHCA matrix (A, C) and in linear positive mode using DHB matrix (B, D). Samples were prepared after digestion using the neutralization method followed by desalting with cotton HILIC SPE microtips prepared from three different brands of cotton wool pads (A, B). The resulting profiles were compared with the pattern of glycoforms purified by Sepharose beads or cotton HILIC SPE microtips after drying by vacuum centrifugation, and digestion with sequencing grade trypsin (C, D). For each independent experiment, relative intensities and standard deviations (STD) were calculated from 8 replicates. Glycan code as in Figure 1 of Chapter 1.

HILIC micro-SPE, and MALDI-TOF-MS analysis. This procedure was repeated on three different days. Mass spectrometric analyses were performed in reflectron mode and linear mode, demonstrating that both IgG1 (Figure 3A and B) and IgG2 (Figure S-2A and B) Fc N-glycosylation profiles could be registered with low intraday and interday variability. Next to the hitherto used cotton material, two other brands of cotton wool pads were used for HILIC SPE microtip preparation and IgG Fc N-glycosylation profiling. All three brands of cotton wool pads provided very similar results for both IgG1 (Figure 3A and B) and IgG2 (Figure S-2A and B). Moreover, the method was compared to a previously described approach.¹⁴⁸ The major differences compared to the previous method are that (1) drying of Protein A eluates by vacuum centrifugation was replaced for a neutralization step, (2) sequencing grade trypsin was replaced by TPCK-treated trypsin, and (3) cotton HILIC micro-SPE was performed instead of 96-well plate HILIC SPE using Sepharose beads. A third approach combined the previously described sample preparation¹⁴⁸ (i.e. protein A capturing, drying by vacuum centrifugation, and cleavage of IgG with sequencing grade trypsin) with cotton HILIC micro-SPE purification and reflectron mode MALDI-TOF-MS analysis. With all different protocols, very similar IgG1 (Figure 3C and D) and IgG2 (Figure S-2C and D) Fc N-glycosylation profiles were obtained by reflectron mode (Figure 3A and C and Figure S-2A and C) and linear mode MALDI-TOF-MS (Figure 3B and D and Figure S-2B and D).

In order to evaluate the suitability of cotton microtips for the purification of glycoconjugates with a high level of sialylation, we evaluated the performance of cotton HILIC micro-SPE and 96-well plate Sepharose HILIC SPE for glycopeptide extraction from a tryptic digest of bovine fetuin (5 µg). Reverse phase nanoLC-MS analyses of the HILIC micro-SPE (Figure 4B) and Sepharose (Figure 4C) eluates were dominated by glycopeptide signals and showed a glycopeptide pattern very similar to the one observed for the total fetuin tryptic digest (Figure 4A). Glycopeptides were identified and assigned based on MS and MS/MS data (Figure 4D). Non-glycosylated fetuin peptides were efficiently removed by both HILIC procedures: for example, fetuin peptides E₂₉PACDDPDTEQAALAAVDYINK₅₀ (at m/z 802.7 and 1203.6 in triple and double charged form; Figure 4A) and Q₁₂₁DGQFSVLFTK₁₃₁ (native at m/z 1269.7 and with pyroglutamate formation at m/z 1252.6, $[M+H]^+$; Figure 4A) were absent from the HILIC eluates (Figure 4B and C). Together with the results obtained for IgG, these data show that the two HILIC SPE methods employing Sepharose and cotton microtips are equally suitable for enriching sialylated as well as non-sialylated glycopeptides with both small and large peptide moieties.

Glycan purification using cotton HILIC SPE microtips. As another field of application we tested the use of cotton HILIC SPE microtips for sample preparation of PNGase-F released glycans for MALDI-TOF-MS. N-glycans were enzymatically released from human IgG and human transferrin samples containing detergents (SDS, NP-40) and salts (PBS). Both neutral and acidic (sialylated) N-glycans were detected by MALDI-TOF-MS

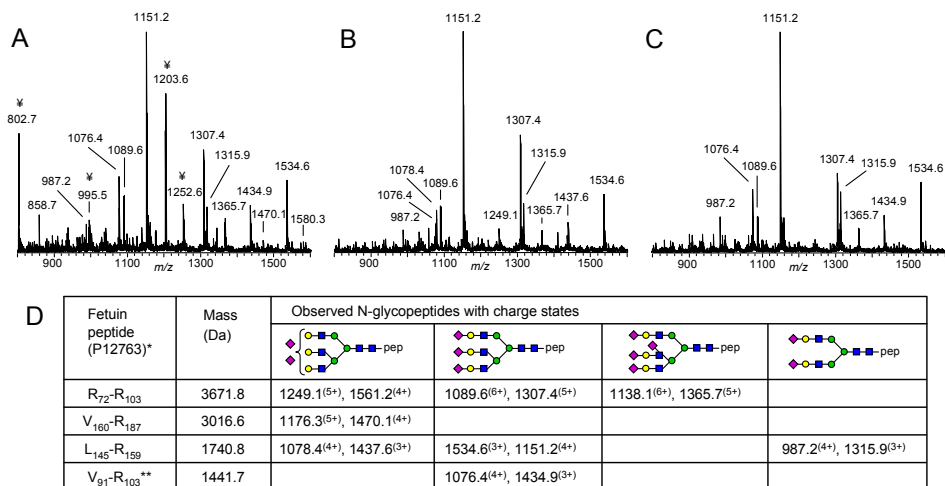


Figure 4. Reverse phase nanoLC-MS analysis of a tryptic fetuin digest without purification (A) and after additional HILIC purification by cotton micro-SPE (B) or Sepharose (C). Tryptic glycopeptides covering all three N-glycosylation sites of fetuin were observed (D). ^{173,174} *, Fetuin SwissProt entry number; †, signals related from peptides; **, cleavage between H and V¹⁷⁵; glycan code as in Figure 1 of Chapter 1.

after cotton HILIC micro-SPE purification whilst direct MALDI-TOF-MS analysis of the glycan release samples without SPE purification did not allow registration of N-glycans but was dominated by detergent clusters (Figure S-3). Hence, these experiments demonstrated the successful clean-up of N-glycan release samples for MALDI-TOF-MS analysis by removing detergents and salts with cotton HILIC SPE microtips.

Cotton HILIC SPE microtip capacity and recovery. To evaluate the capacity and recovery of the developed cotton SPE microtips, a dilution series of 2-AA labeled maltoheptaose was prepared with and without cotton micro-SPE and analyzed by HPLC-Flu. Initially, the 2-AA labeled maltoheptaose dilution series was prepared with the original cotton micro-SPE conditions using 83% acetonitrile. While this revealed a linear relation over the entire dilution curve (data not shown) recoveries were low (average recovery ~ 40%). By increasing the acetonitrile concentration to 90% the 2-AA labeled maltoheptaose recoveries could substantially be increased (average recovery 82% when employing 2-AA labeled maltoheptaose amounts of up to 2.5 µg (Figure S-4). When 10 µg of 2-AA labeled maltoheptaose were used, a recovery of approximately 50% was observed, indicating that the capacity is at least 5 µg 2-AA labeled maltoheptaose per 500 µg cotton. In addition, graphitized carbon nanoLC-MS¹⁷⁶ was employed to analyze the cotton HILIC micro-SPE efficacy of native (unlabeled) maltoheptaose resulting in an average recovery of 94% when applying up to 2.5 µg oligosaccharide (data not shown).

Discussion

We here established cotton wool HILIC microtips for the purification of glycopeptides with various degrees of sialylation and peptide moieties of different sizes. In addition, cotton HILIC micro-SPE was applied to purify neutral and sialylated N-glycans released by PNGase-F followed by mass spectrometric detection. Similar to Poly HEA and carbohydrate HILIC stationary phases such as Sepharose and microcrystalline cellulose, cotton wool is a non-ionic, neutral stationary phase, and HILIC retention is expected to be caused solely by hydrogen bonding.¹³⁹ By contrast, ionic interactions may contribute to HILIC retention on ZIC-HILIC as well as amine-based stationary phases, which may be modulated by the addition of salt and/or ion-pairing reagents.^{138,139}

Cotton wool microtips are cheap and can easily and quickly be prepared in every lab. Microtips have been introduced with various stationary phases,^{177,178} including ZIC-HILIC microtips.¹⁷⁹ In contrast to some other microtips,^{178,179} the cotton stationary phase stays in position, both with liquid aspiration and dispensation. Moreover, the stationary phase appeared to be compatible with acidic and high acetonitrile conditions.

Raw cotton is mainly composed of cellulose (over 90%).¹⁸⁰ Cotton is used in a variety of commercial products such as clothing, cotton swabs (q-tips) and cotton wool pads. For the manufacturing of cotton wool for pads and q-tips, the raw cotton is subjected to extensive bleaching after which the fibers are carded, randomized and treated with water at high pressure to cross and tie the fibers. During the manufacturing process traces of wax and proteins are removed from cotton, and as a result cotton wool pads are composed of virtually pure cellulose.

The cotton wool HILIC micro-SPE procedure was used for the extraction of Fc N-glycopeptides from human tryptic plasma IgG digests followed by MALDI-TOF-MS analysis. The method showed good repeatability and did not appear to depend on a specific brand or batch of cotton wool pads. The results were very similar to those obtained previously after 96-well plate sample preparation of IgG glycopeptides using either reverse phase-SPE desalting, or HILIC SPE purification with Sepharose and microcrystalline cellulose.¹⁴⁸ When analyzed in reflectron mode MALDI-TOF-MS, no registration of sialylated glycopeptides was achieved due to loss of sialic acids by in-source and metastable decay. By contrast, in linear mode MALDI-TOF-MS the lack of an extraction delay strongly reduced in-source decay, and metastable decay products are not resolved from their precursor ions, allowing detection of sialylated glycopeptides.

Next to establishing the HILIC SPE microtips, we introduce two additional modifications to a previously described IgG Fc N-glycosylation profiling protocol.¹⁴⁸ First, tryptic cleavage was performed using TPCK-treated trypsin instead of the more expensive sequencing-grade trypsin. Second, the rather laborious vacuum centrifugation step, which was applied to remove formic acid from the Protein A eluates, has been substituted by a simple neutralization, making the protocol easier and more suitable for automation.

The here described cotton wool microtips are convenient devices for simple and fast sample preparation of tryptic IgG digests, and allow the determination of IgG Fc N-glycosylation features such as galactosylation, sialylation, fucosylation, and incidence of bisecting N-acetylglucosamine by MALDI-MS analysis. IgG Fc N-glycosylation has an important biological role in modulating both antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity.¹⁸¹ Hence, IgG Fc N-glycosylation of biotechnologically produced IgGs is being designed in order to maximize the efficacy in e.g. anti-cancer therapy (next generation therapeutic antibodies). Cotton wool microtips are particularly convenient tools for the preparation of low amounts of samples for mass spectrometric analysis and may be applied in (1) IgG Fc N-glycosylation profiling of clinical samples which often show disease-associated IgG glycosylation changes,^{15,22,24,67,147} and (2) analysis of recombinantly expressed IgG.

Carbohydrate-based stationary HILIC phases have previously been used for total plasma N-glycome analysis by mass spectrometry.^{85,94} 2-AA labeled N-glycans are purified from the labeling mix which contains excess label, reducing agent, various salts, plasma lipids, and large amounts of detergents together with denatured proteins, followed by glycan detection using MS.^{85,94} Notably, cotton wool microtips are likewise suitable for removal of salts and detergents after enzymatic N-glycan release which, together with the high capacity and favorable SPE elution conditions, resulted in efficient mass spectrometric detection of N-glycans (Figure S-3).

Cotton wool microtips may serve as sample clean-up devices in glycomics and proteomics applications in which denaturants and surfactants are often added to improve protein solubility and proteolytic cleavage.^{143,182-186} Moreover, we expect that the tips could provide a valuable tool for the enrichment of other hydrophilic molecules as well. Minor modifications of the here presented protocol may, however, be required. While cotton wool HILIC microtips appear to be a good choice for processing low numbers of samples, they may be applicable to larger numbers of samples by using multi-channel pipettes or by transferring the SPE method to a robotic platform.

Acknowledgements

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Supporting information

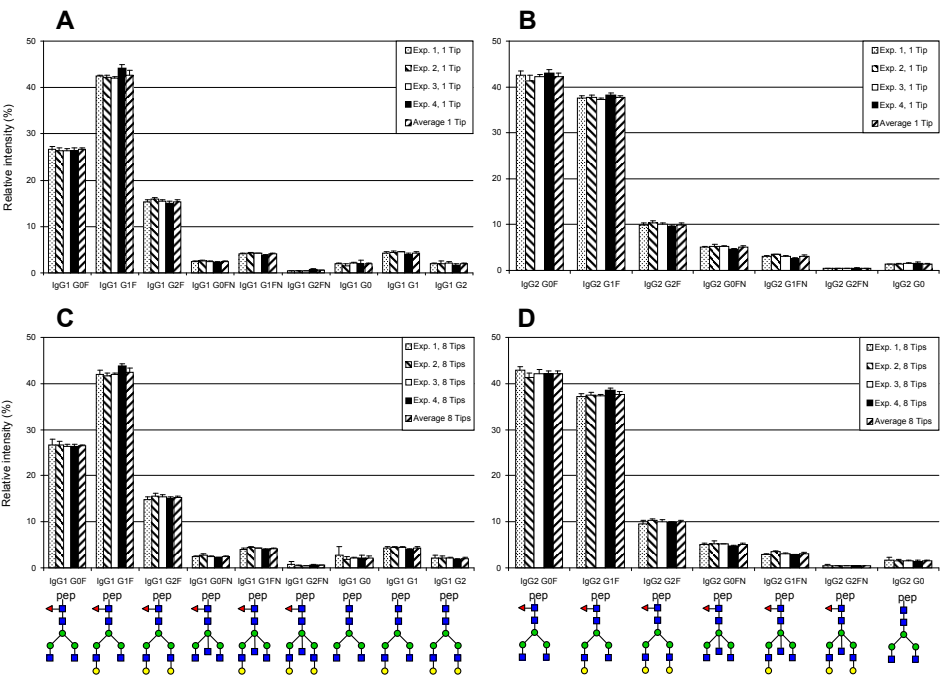


Figure S-1. Repeatability of cotton HILIC microtips for desalting and purification of IgG glycopeptides. Analysis was performed by reflectron mode MALDI-TOF-MS with CHCA matrix. A tryptic IgG digest pool was desalted 8 times either with one cotton HILIC microtip (A and B) or with eight different cotton HILIC microtips (C and D). The experiment was repeated on four different days. Glycan code as in Figure 1 of Chapter 1.

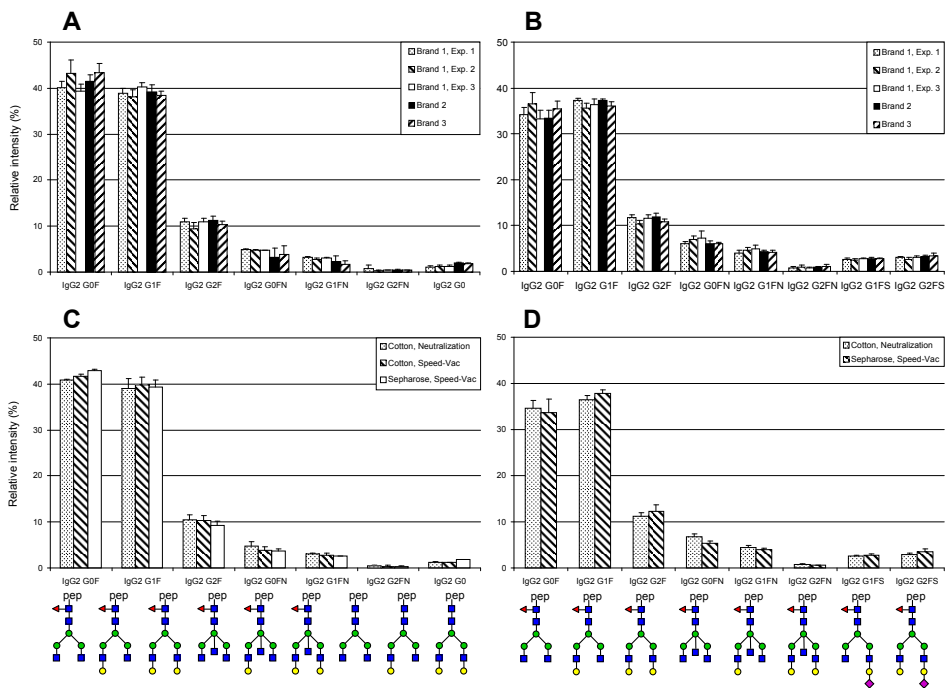


Figure S-2. Repeatability of IgG2 glycopeptide profiling applying cotton HILIC SPE microtips. IgG2 glycopeptides were detected by MALDI-TOF-MS in reflectron mode using CHCA matrix (A, C) and in linear mode using DHB matrix (B, D). Samples were prepared after digestion using the neutralization method followed by desalting with cotton HILIC SPE microtips prepared from three different brands of cotton wool pads (A, B). The resulting profiles were compared with the pattern of glycoforms purified by Sepharose beads or cotton HILIC SPE microtips after drying by vacuum centrifugation, and digestion with sequencing grade trypsin (C, D). For each independent experiment relative intensities and STDs were calculated from 8 replicates. Glycan code as in Figure 1 of Chapter 1.

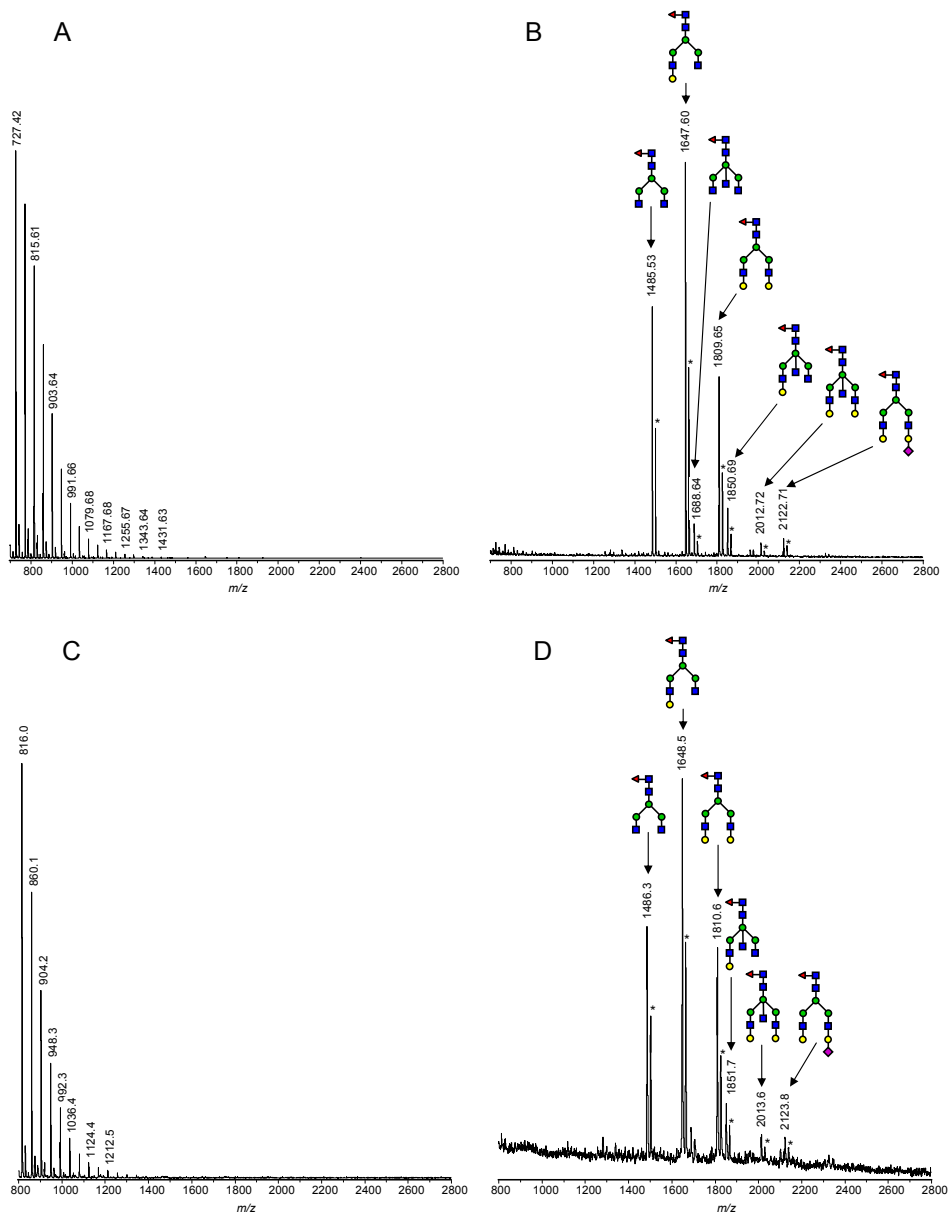


Figure S-3. MALDI-TOF-MS of N-glycans after cotton HILIC micro-SPE purification. N-glycans enzymatically released from IgG (A-F) and human transferrin (G, H) were analyzed by MALDI-TOF-MS in the reflectron positive mode (A, B), linear positive mode (C, D), and linear negative mode (E-H) without (left panels: A, C, E, G) and with (right panels: B, D, F, H) purification by cotton HILIC micro-SPE. Glycans were registered as sodium adducts in positive-ion mode and as deprotonated species in negative-ion mode. *, Potassium adducts; glycan code as in Figure 1 of Chapter 1.

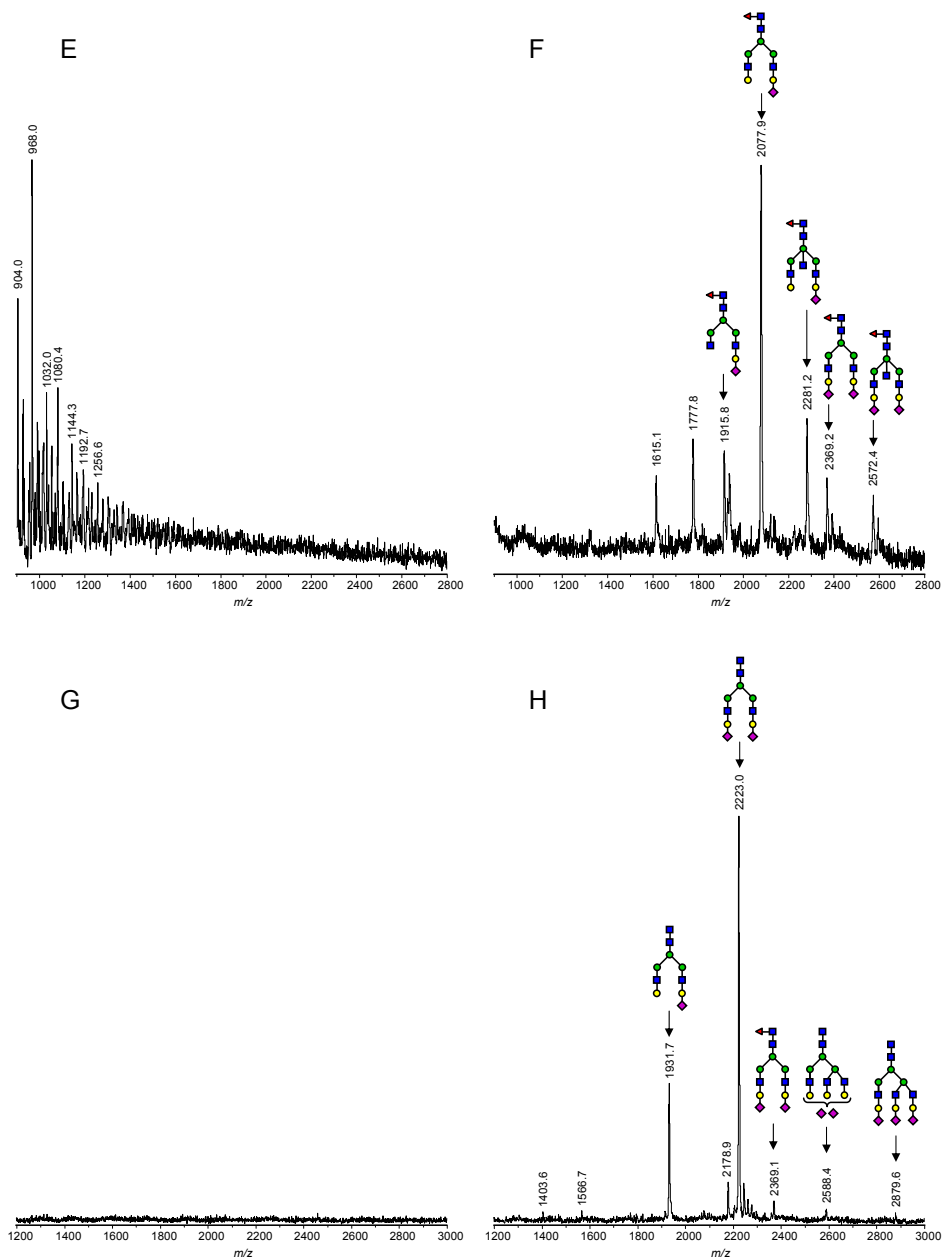


Figure S-3. Continued.

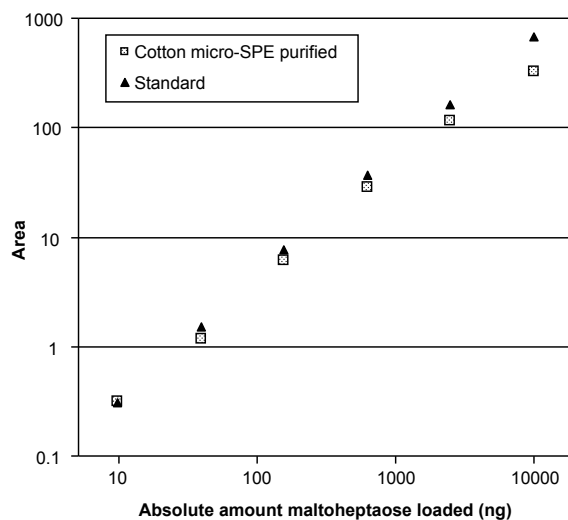


Figure S-4. Cotton HILIC microtip capacity and recovery. HILIC-Flu analysis of a 2-AA labeled maltoheptaose standard dilution series performed with (squares) and without (triangles) cotton SPE.

Immunoglobulin G glycopeptide profiling by matrix-assisted laser desorption ionization Fourier transform ion cyclotron resonance mass spectrometry

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Abstract

Immunoglobulin G Fc glycosylation is essential for Fc γ -receptor mediated activities. Changes in IgG Fc glycosylation have been found to be associated with various diseases. Here we describe a high-throughput IgG glycosylation profiling method. Sample preparation is performed in the 96-well plate format: IgGs are purified from 2 μ l of human plasma using immobilized Protein A. IgGs are cleaved with trypsin, and the resulting glycopeptides purified by reverse phase or hydrophilic interaction solid-phase extraction. Glycopeptides are analyzed by intermediate pressure MALDI-FTICR-MS. Notably, both DHB and CHCA matrices allowed the registration of sialylated as well as non-sialylated glycopeptides. Data were automatically processed, and IgG isotype-specific Fc glycosylation profiles were obtained. The entire method showed an interday variation below 10% for the 6 major glycoforms of both IgG1 and IgG2. The method was found suitable for isotype-specific high-throughput IgG glycosylation profiling from human plasma. As an example we successfully applied the method to profile the IgG glycosylation of 62 human samples.

Introduction

In human polyclonal immunoglobulin Gs (IgG) are present in high abundances (total concentration ca. 10 mg/ml plasma). Each IgG molecule consists of 4 polypeptide chains, i.e., two heavy chains and two light chains. IgGs occur in four subclasses (IgG1, IgG2, IgG3, and IgG4)⁴ which all carry N-glycans at asparagine 297 in the conserved C_H2 region of the fragment crystallizable (Fc) part of their heavy chains. Fc N-glycans of human IgG are bi-antennary complex-type structures which often carry a core-fucose.¹⁴⁷ The N-glycans vary in the number of galactose residues and are referred to as G0F (no galactoses), G1F (one galactose), and G2F (2 galactoses). A small portion of the N-glycans may contain a sialic acid residue on the antennae and/or a bisecting N-acetylglucosamine.¹⁴⁷

The biological activity of IgG is modulated by its Fc N-glycosylation. First, lack of core-fucoses on the Fc N-glycans of IgG1 may lead to a drastic enhancement of antibody-dependent cellular cytotoxicity (ADCC).^{43,44} Therefore, glycosylation of therapeutic antibodies is often engineered to achieve lower core-fucosylation and high ADCC activity.^{5,46} Second, as shown in a mouse arthritis model^{35,47,48} the anti-inflammatory properties of intravenous immunoglobulin are dependent on sialylation of the Fc N-glycans. For a variety of diseases¹⁴⁷ such as rheumatoid arthritis,^{3,14} Crohn's disease,^{21,27} and HIV infection³¹ IgG galactosylation has been shown to be low. Moreover, in healthy individuals age, gender, and pregnancy are reflected by IgG glycosylation features.^{4,8-10,13,14}

IgG glycosylation analysis is important for the characterization of monoclonal therapeutic antibodies as well as the determination of glycosylation changes of polyclonal IgGs as part of humoral immune responses. Two potential strategies to determine IgG Fc N-glycosylation are analysis of glycopeptides or released N-glycans by mass spectrometry (MS).^{77,147} Although both strategies provide valuable information regarding N-glycan heterogeneity, only IgG glycopeptide analysis allows discrimination between different IgG subclasses and provides N-glycan profiles that are Fc specific.⁴ Analysis of IgG glycopeptides is often performed by reverse phase liquid chromatography (RP-HPLC)^{4,50,79} or hydrophilic interaction liquid chromatography (HILIC)⁹² coupled to MS. LC-MS analyses provide very detailed IgG Fc glycosylation profiles, yet these methods suffer from relatively long analysis times and extensive data processing. Another strategy to determine IgG glycopeptide microheterogeneity involves analysis by matrix-assisted laser desorption (MALDI)-MS.^{77,102,119} While MALDI-MS is inherently suited for high-throughput analysis, complementation with high-throughput sample preparation and data processing techniques is required for high-throughput IgG glycosylation analysis. Other challenges in automated, high-throughput MALDI-MS analysis include controlled matrix crystallization and minimization of in-source and metastable decay in order to retain the labile sialic acid groups.

Here, we address these challenges and describe the development, validation, and application of a high-throughput IgG glycosylation profiling method by intermediate pressure MALDI Fourier transform ion cyclotron resonance (FTICR) MS of glycopeptides. The entire high-throughput sample preparation procedure (IgG capturing, cleavage, desalting and purification) is performed at the 96-well plate level. The developed method was applied to IgG Fc glycopeptide profiling of 62 human samples.

Experimental section

IgG purification. IgGs were affinity captured from total human serum or plasma according to Wuhler *et al.* 2007, with minor modifications.⁴ Briefly, rProtein A-Sepharose™ Fast Flow beads (90 µm; GE Healthcare, Eindhoven, the Netherlands) were washed three times with 10 volumes of PBS. To each well of a 96-well filter plate (Multiscreen Solvinert, 0.45 µm pore-size low-binding hydrophilic PTFE; Millipore, Billerica, MA), 50 µl of slurry containing ca. 15 µl beads, 150 µl PBS and 2 µl serum were applied. The plate was sealed with adhesive tape and incubated on a shaker for 1 hour at room temperature. After incubation, the beads were washed on a vacuum manifold with 3x 200 µl PBS and 3x 200 µl water. Captured IgGs (IgG1, IgG2, and IgG4) were eluted into a V-bottom microtitration plate with 100 µl of 100 mM formic acid (Fluka, Steinheim, Germany). Samples were dried by vacuum centrifugation before trypsin digestion.

Trypsin digestion. Captured IgGs were dissolved in 20 µl of 50 mM ammonium bicarbonate buffer (Fluka) containing 15% acetonitrile (ACN; Biosolve BV, Valkenswaard, the Netherlands), and shaken for 10 minutes. To minimize potential autolysis, lyophilized sequencing grade modified trypsin (Promega, Madison, WI) was first dissolved in ice-cold 20 mM acetic acid (Merck, Darmstadt, Germany) to a final concentration of 0.2 µg trypsin per µl. Trypsin was further diluted to 0.01 µg trypsin per µl with ice-cold 15% ACN, and immediately 20 µl was added to the IgG samples. Samples were shaken for 1 minute, checked for the correct pH (>6) and incubated overnight at 37°C. Glycopeptides were desalted and purified by 96-well solid phase extraction (SPE) with RP or HILIC.

RP-SPE. For RP desalting and purification, 5 mg Chromabond C18_{end} material (45 µm; Macherey-Nagel, Düren, Germany) was applied to each well of an OF1100 96-well polypropylene filter plate with a 10 µm polyethylene frit (Orochem Technologies Inc., Lombard, IL). The RP stationary phase was activated and conditioned with 3x 200 µl 80% ACN containing 0.1% trifluoroacetic acid (TFA; Fluka) and 3x 200 µl 0.1% TFA, respectively. Whole IgG digests (40 µl) were diluted 10x in 0.1% TFA, loaded onto the C-18 beads, and washed with 3x 200 µl 0.1% TFA. The entire procedure was performed on a vacuum manifold under slight pressure reduction of ca. 3 mbar. IgG (glyco)

peptides were eluted into a V-bottom microtiter plate by centrifugation at 500 rpm with 60 μ l of 18% ACN containing 0.1% TFA. Samples were dried by vacuum centrifugation, reconstituted in 60 μ l water and stored at -20°C until analysis by MALDI-MS.

HILIC-SPE. For HILIC desalting and purification, 5 mg cellulose microcrystalline particles for column chromatography (Merck) or 5 μ l Sepharose Cl-4B beads (45-165 μ m; GE Healthcare, Uppsala, Sweden) were applied to each well of a 96-well filter plate (Multiscreen Solvinert, 0.45 μ m pore-size low-binding hydrophilic PTFE; Millipore, Billerica, MA). The HILIC stationary phases were activated and conditioned with 2x 200 μ l of water and 2x 200 μ l 83% ACN, respectively. Whole IgG digests (40 μ l) were diluted with ACN to a final concentration of 83% ACN, loaded to the HILIC stationary phases and shaken for 5 minutes. SPE activation, conditioning and sample loading were performed on a vacuum manifold under slight pressure reduction of ca. 3 mbar. The captured IgG glycopeptides were washed and eluted at 170 mbar vacuum with 3x 200 μ l 83% ACN with 0.1% TFA, 3x 200 μ l 83% ACN and 3x 30 μ l water, respectively. All samples were stored at -20°C until MALDI-MS analysis.

Preparation of MALDI samples. For MALDI-MS analysis, purified and desalted tryptic (glyco)peptides (1 μ l) were spotted onto polished stainless steel or AnchorChip 600/384 MALDI plates (Bruker Daltonics, Bremen, Germany). Samples spotted onto AnchorChip MALDI plates were overlaid with 1 μ l dihydroxybenzoic acid (DHB, 5 mg/ml 50% ACN with 0.1% TFA), and samples spotted onto polished stainless steel MALDI plates were overlaid with 1 μ l α -cyano-4-hydroxycinnamic acid (CHCA, 5 mg/ml 50% ACN). The AnchorChip plates were covered with a pierced cap containing 5 holes of ca. 5 mm (i.d) and both matrixes were allowed to dry in air at room temperature. Samples were analyzed on an Ultraflex II MALDI-TOF/TOF-MS and on an APEX-ultra 9.4 tesla FTICR-MS (Bruker Daltonics).

MALDI-TOF-MS. Analysis by MALDI-TOF-MS was performed in the reflectron positive and linear positive mode. The samples were irradiated by a smartbeam™ 200 Hz solid-state laser. The acceleration voltage was 25 kV, and the ions between m/z 1300-4600 were recorded. Each mass spectrum was generated by summing the ions of 2000 laser shots. To allow homogeneous spot sampling a random walk laser movement with 50 laser shots per raster spot was applied. The laser intensity was optimized to give the best signal-to-noise ratio and resolution for each sample. During automatic MALDI-TOF-MS the masses between 2000-3500 Dalton (Da) were evaluated after every set of 100 laser shots. Evaluation in the reflectron mode was performed by a SNAP peak detection algorithm with a Signal to Noise Threshold of 6, Quality Factor Threshold of 50 and a TopHat baseline subtraction. During linear mode MALDI-TOF-MS evaluation was performed by a centroid peak detection algorithm with a Signal to Noise Threshold of 3, a Peak Width of 1 m/z , a Height of 80%, and a TopHat baseline subtraction. All sets of 100 laser shots not fitting these criteria were not added to the sum spectrum. All data processing and evaluation were performed with flexAnalysis

Software (Bruker Daltonics) and Microsoft Excel, respectively. Typically, reflectron positive and linear positive spectra were calibrated internally followed by peak picking using the interactive SNAP and interactive centroid algorithm, respectively.

MALDI-FTICR-MS. The 9.4 tesla FTICR APEX-ultra mass spectrometer was equipped with a dual ESI/MALDI ion source (Apollo II) incorporating a quadrupole mass filter and a smartbeam™ laser system. The MALDI source is at intermediate pressure enabling the detection of the labile glycosylated peptides.¹⁴⁴ All experiments used a laser spot size of approximately 150 μm , laser fluence slightly above threshold and a laser repetition rate of 200 Hz. The quadrupole was operated in RF-only mode with the selection mass set to m/z 2500, thus ejecting all ions below m/z 1800. The MALDI-FTICR-MS experiments utilized a customized experiment sequence (the pulse program). Briefly, the ions produced from 50 laser shots were accumulated in a hexapole and then transferred through the RF-only quadrupole to the collision cell. The sample stage was then moved 200 μm and fresh sample interrogated with the next 50 laser shots. This cycle was performed 9 times, effectively accumulating ions from 450 laser shots in the collision cell. The accumulated ions were then transferred to the ICR cell for a mass analysis scan. Each spectrum is the sum of 8 such scans. All data were acquired using Bruker ApexControl 3.0.0 software in expert mode. Mass spectra were internally calibrated using a list of known glycopeptides and Bruker DataAnalysis 3.4 in batch mode. After calibration, the spectra were exported to an ASCII format consisting of pairs of m/z and intensity values in two columns. A list of pre-defined features were then extracted from each spectrum and merged to a complete data matrix using the in-house developed software “Xtractor”. As input, Xtractor takes a mass spectrum in the ASCII format and a list of pre-defined peaks or m/z windows. The program then integrates the peaks by summing all intensities in the defined windows. The software is freely available at www.ms-utils.org/Xtractor. The complete sample-data matrix was finally evaluated using Microsoft Excel.

MALDI-FTICR-MS glycopeptide profiling of a human sample cohort. Twenty human sera (12 male, 8 female) and 42 human citrate plasma samples (20 male, 22 female) were obtained from volunteers ranging in age between 22 and 79 years. As no selection on the health status was performed, prevalence of diseases which are known to interfere with IgG glycosylation (autoimmune diseases, inflammatory diseases, and cancer)¹⁴⁷ is assumed to be low within the cohort. Human polyclonal IgGs were purified by Protein A affinity chromatography and digested overnight with trypsin. The resulting glycopeptides were desalted by Sepharose SPE, spotted on a polished stainless steel MALDI target plate, overlaid with CHCA, and analyzed by MALDI-FTICR-MS. Spectra were processed automatically and data evaluation was performed using Microsoft Excel and SPSS 16.0.

Results

IgG glycopeptide profiling by MALDI-MS. To evaluate the potential of MALDI-MS for high-throughput profiling of IgG glycosylation at the glycopeptide level, human polyclonal IgGs were captured from the plasma of a volunteer using immobilized protein A. The captured IgGs were digested overnight with trypsin in the presence of ACN to ensure maximum trypsin activity. Desalting and purification of the tryptic glycopeptides was performed by C18 SPE, and the IgG glycopeptides were spotted, overlaid with matrix and analyzed by different MALDI-MS techniques. First, the N-glycopeptide profiles were determined by MALDI-TOF-MS in the linear positive mode. To this end, IgG glycopeptides were spotted onto an AnchorChip target plate and overlaid with DHB. The target plate was covered with a pierced cap to slow down and control the drying process. This resulted in a dense and rather homogeneous preparation of DHB crystals and improved the spot to spot reproducibility during automatic measurement. The profiles obtained represent the tryptic N-glycopeptide microheterogeneity of IgG1, 2 and 4 subclasses (Figure 1A). Structural assignment of the detected glycoforms was performed on the basis of literature knowledge of IgG N-glycosylation.^{3,4,9,10,79,148} The most abundant signals were obtained for the glycopeptides of IgG2 followed by IgG1 and IgG4. Sialylated glycopeptides were detected next to species with neutral glycoforms. Isotopic resolution could not be achieved with linear mode MALDI-TOF-MS. Second, the IgG glycopeptide profile was determined with reflectron positive MALDI-TOF-MS using DHB (data not shown) and CHCA matrix (Figure 1B). The two matrices resulted in similar IgG glycopeptide profiles with isotopic resolution. IgG glycopeptide profiles were similar to those obtained with linear positive MALDI-TOF-MS, except sialylated glycopeptides could not be detected in the reflectron mode. Third, high mass resolution detection glycopeptides was achieved by MALDI-FTICR-MS using DHB and CHCA as matrix (Figure 1C and D). The DHB overlaid samples provided signals for all glycopeptides including the sialylated species which were similar to those obtained during linear positive MALDI-TOF-MS. Notably, the samples prepared with CHCA also allowed the detection of sialylated glycopeptides (Figure 1D), albeit at a lower relative intensity than with the DHB matrix (Figure 1C). A wide range of Fc N-glycopeptides could unambiguously be assigned to IgG1, 2 and 4 by high resolution MALDI-FTICR-MS (Table 1). Spectra were characterized by a particularly good signal-to-noise ratio.

In conclusion, the different MALDI-MS techniques provided similar IgG glycopeptide profiles. Major differences were only observed for sialylated glycopeptides, which were not observed by MALDI-TOF-MS in the reflectron positive mode. The high resolution, excellent signal-to-noise ratio, and the possibility to profile both sialylated and non-sialylated glycopeptides independent of the MALDI matrix used, made intermediate pressure MALDI-FTICR-MS extremely suited for IgG glycopeptide profiling.

Table 1. Tryptic glycopeptides of human IgG subclasses 1, 2 and 4.

Glycan species	IgG1 P01857 ^c [M+H] ⁺	IgG2 P01859 ^c [M+H] ⁺	IgG4 P01861 ^c [M+H] ⁺
no glycan	1189.5120	1157.5222	1173.5171
G0F ^d	2634.0459 ^{b1}	2602.0561	2618.0510 ^{a1}
G1F	2796.0987 ^{b2}	2764.1089	2780.1038 ^{a2}
G2F	2958.1515	2926.1617	2942.1566
G0FN	2837.1253 ^{b3}	2805.1355	2821.1304 ^{a3}
G1FN	2999.1781 ^{b4}	2967.1883	2983.1832 ^{a4}
G2FN	3161.2309	3129.2411	3145.2360
G1FS	3087.1941 ^{b5}	3055.2043	3071.1992 ^{a5}
G2FS	3249.2469	3217.2571	3233.2520
G0	2487.9880	2455.9982	2471.9931
G1	2650.0408	2618.0510 ^{a1}	2634.0459 ^{b1}
G2	2812.0936	2780.1038 ^{a2}	2796.0987 ^{b2}
G0N	2691.0673	2659.0775	2675.0724
G1N	2853.1202	2821.1304 ^{a3}	2837.1253 ^{b3}
G2N	3015.1730	2983.1832 ^{a4}	2999.1781 ^{b4}
G1S	2941.1362	2909.1464	2925.1413
G2S	3103.1890	3071.1992 ^{a5}	3087.1941 ^{b5}

^{a1} – ^{a5} isomeric glycopeptide species of IgG4 and IgG2.

^{b1} – ^{b5} isomeric glycopeptide species of IgG1 and IgG4.

^c SwissProt entry number

^d glycan structural features are given in terms of number of galactoses (G0, G1, G2), fucose (F), bisecting N-acetylglucosamine (N), and N-acetylneuraminic acid (S).

Repeatability MALDI mass analyzers. To determine the repeatability of the various MALDI mass analyzers, the IgG glycopeptide sample described above was spotted eight times onto an AnchorChip target plate and a polished stainless steel target plate, overlaid with matrix and analyzed by linear positive MALDI-TOF-MS (DHB), reflectron positive MALDI-TOF-MS (CHCA) and MALDI-FTICR-MS (DHB and CHCA). This experiment was performed three times each on different days. Mass spectra were processed automatically which involved internal calibration, baseline subtraction (MALDI-TOF only), and peak picking. The best interday repeatability was observed for the MALDI-TOF-MS techniques, providing less than 5% relative standard deviation (RSD) of the normalized peak heights for the 6 major glycoforms of both IgG1 and IgG2 independent of the matrix used (data not shown). For these major glycoforms, the interday repeatability test with MALDI-FTICR-MS, gave RSDs below 10% with both matrices (data not shown).

Sample preparation by RP-SPE. Desalting and purification of tryptic IgG glycopeptides was performed using in house packed 96-well C-18 plates to allow high-throughput

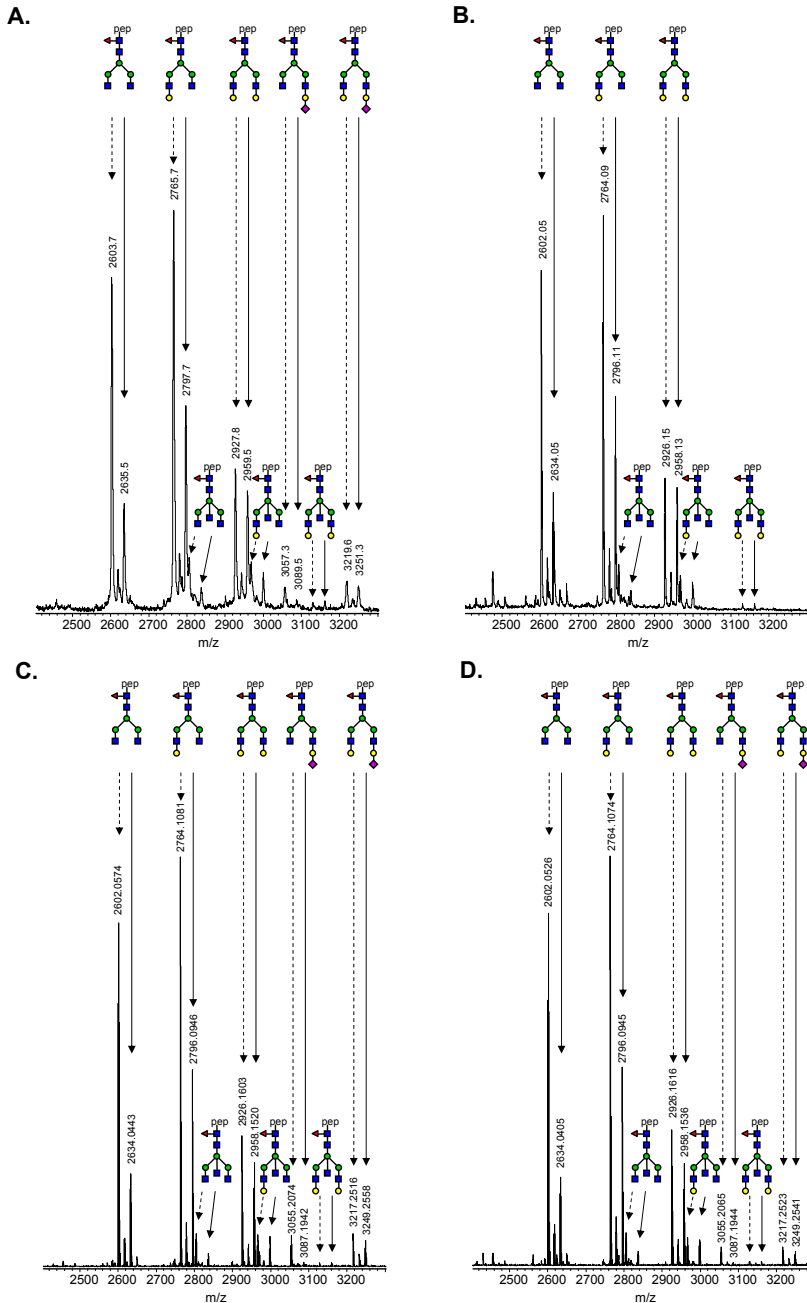


Figure 1. Comparison of four MALDI-MS methods for profiling of purified tryptic IgG Fc glycopeptides. Profiling was performed after RP-SPE desalting by linear mode MALDI-TOF-MS with DHB (A), reflectron mode MALDI-TOF-MS using CHCA (B), and MALDI-FTICR-MS using DHB (C) and CHCA (D). Continued arrows, IgG1 glycopeptides; dashed arrows, IgG2 glycopeptides; glycan code as in Figure 1 of Chapter 1.

sample preparation. The IgG glycopeptides were eluted from the C-18 material with low concentrations of ACN in the presence of TFA, to minimize co-elution of interfering peptides. The robustness and repeatability of the entire analytical method including sample preparation (protein A affinity, digestion and C-18 SPE) and mass spectrometry was assessed by performing three independent experiments comprising eight replicates each. In all three experiments, the eight replicates were spotted both on an AnchorChip and on a polished stainless steel target plate, overlaid with matrix, and analyzed by the different MALDI-MS techniques. Within the three experiments, the intraday variability was determined for IgG1 (Figure 2) and IgG2 (Figure S-1). For both IgG1 and IgG2, the RSDs of the normalized peak heights for the 6 major glycoforms were below 5% with MALDI-TOF-MS and below 10% with MALDI-FTICR-MS. Moreover, the interday repeatability was determined by comparing the results of the three experiments (Figure 2 and Figure S-1). Again, the RSDs were below

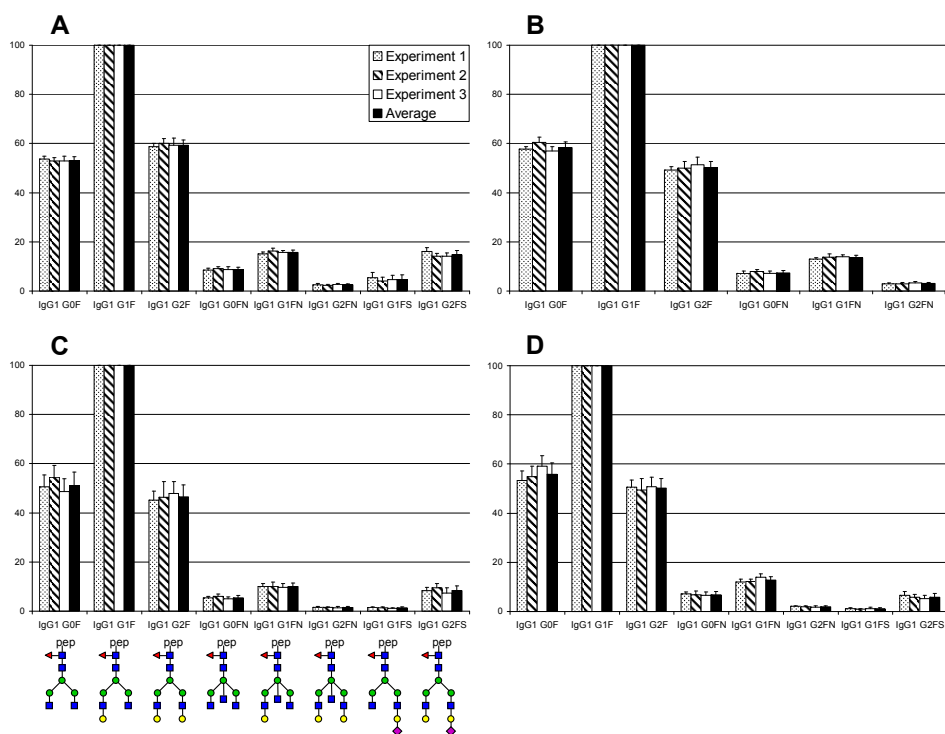


Figure 2. Repeatability of 96-well RP-SPE for desalting and purification of tryptic IgG1 Fc glycopeptides. Analysis was performed by linear mode MALDI-TOF-MS with DHB (A), reflectron mode MALDI-TOF-MS using CHCA (B), and MALDI-FTICR-MS using DHB (C) and CHCA (D). Relative intensities and standard deviations (STD) were determined from three independent experiments comprising eight replicates each. Glycan code as in Figure 1 of Chapter 1.

5% with MALDI-TOF-MS and below 10% with MALDI-FTICR-MS for both IgG1 and IgG2. No difference was observed between the measurement variability and the intra- and interday variability of the sample preparation, independent of the MALDI-MS technique used. This indicated that the variability of the sample preparation was below the 5% measurement variability observed for MALDI-TOF-MS.

Sample preparation by HILIC-SPE. In an effort to simplify the sample preparation for high-throughput IgG glycopeptide profiling, we replaced the RP-SPE step by HILIC-SPE. In the RP-SPE protocol, elution of IgG glycopeptides is achieved with ACN/water in the presence of TFA. To prevent acidic hydrolysis of sialylated glycopeptides and to simplify sample spotting, a 1 to 2 hours drying step by vacuum centrifugation is required to remove the acid. Samples are then dissolved in water. With HILIC-SPE, elution can be achieved with water, thus bypassing the long drying step. The potential of cellulose and Sepharose SPE at the 96-well filter plate level was evaluated for high-throughput IgG glycopeptide desalting and purification. The two HILIC stationary phases were pre-

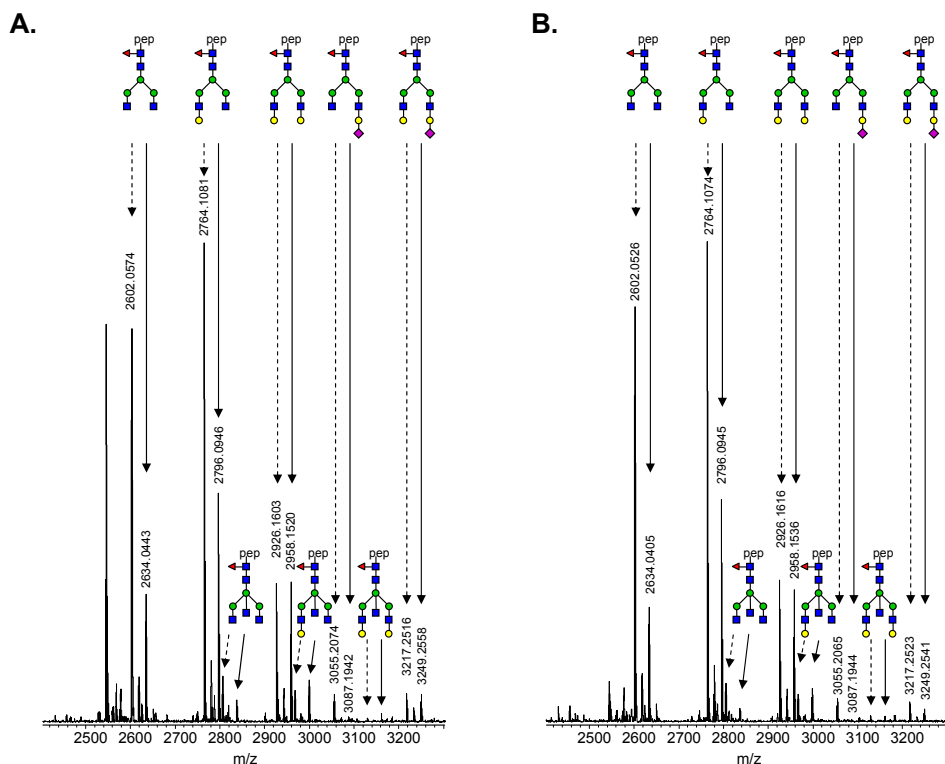


Figure 3. MALDI-FTICR-MS analysis of Sepharose-SPE purified and desalted tryptic IgG glycopeptides using DHB (A) and CHCA (B) as matrix substance. Continued arrows, IgG1 glycopeptides; dashed arrows, IgG2 glycopeptides; glycan code as in Figure 1 of Chapter 1.

washed with water and conditioned with 80% ACN. Tryptic IgG digests were diluted with ACN to a final concentration of 80% ACN, loaded onto the two HILIC stationary phases, washed with 80% ACN and eluted with water. Compared to IgG glycopeptide profiles after RP-SPE, lower relative intensities were obtained for agalactosylated and sialylated glycopeptides (data not shown). To determine the reason for this signal reduction the samples were additionally purified by RP-SPE, after HILIC-SPE. This had no effect for the agalactosylated glycopeptides, but it completely restored the relative intensities for all sialylated glycopeptides (data not shown). This indicated that the lower relative intensities for sialylated glycopeptides after HILIC-SPE were caused by mass spectrometric signal suppression.¹⁸⁷ We eliminated the suppression by including an extra washing step with ACN containing 0.1% TFA. Next, we tested different ACN concentrations in the washing steps to avoid potential losses of agalactosylated glycopeptides known to have the lowest retention on HILIC.¹⁴⁰ We found that for both cellulose and Sepharose stationary phases an ACN concentration of 83% in all HILIC

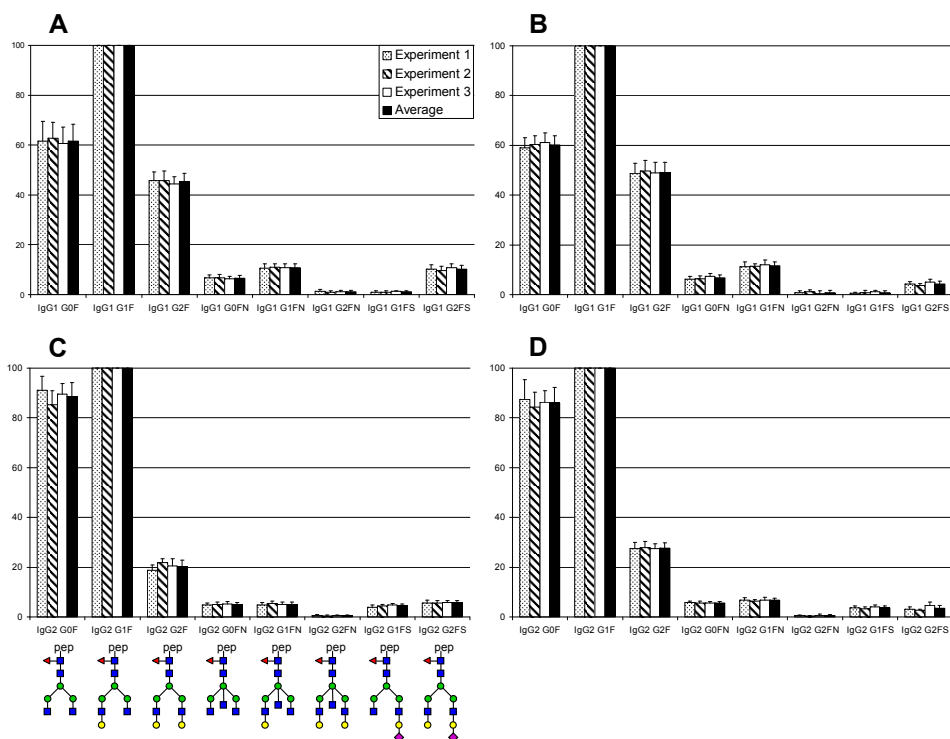


Figure 4. Repeatability of 96-well Sepharose-SPE for desalting and purification of tryptic IgG Fc glycopeptides. Analysis of IgG1 (A and B) and IgG2 (C and D) glycopeptides was performed by MALDI-FTICR-MS using DHB (A and C) and CHCA (B and D). Relative intensities and STDs were determined from three independent experiments comprising eight replicates each. Glycan code as in Figure 1 of Chapter 1.

washing steps consistently resulted in N-glycopeptide profiles (Figure 3 and Figure S-2) similar to those obtained after RP-SPE (Figure 1D).

Robustness and repeatability were similar for HILIC-SPE (Figure 4 and Figure S-3) and RP-SPE (Figure S-1D). Hence, we concluded that RP-SPE followed by the time-consuming vacuum centrifugation and dissolving of the samples in water could be replaced by HILIC-SPE, resulting in a considerable simplification of the protocol. While similar N-glycopeptide profiles were obtained with cellulose and Sepharose SPE, the latter was chosen for further application due to more homogeneous particle sizes which allowed a faster and more constant flow.

IgG glycopeptide profiling of a human sample cohort. The developed technique was used to analyze IgG glycosylation from samples of 62 individuals (32 male and 30 female) in an age range between 22 and 79 years. We chose for 96-well plate HILIC-SPE with Sepharose beads and spotted the samples on a polished stainless steel MALDI target plate. Samples were overlaid with CHCA matrix and analyzed by MALDI-FTICR-MS, and the resulting spectra were processed automatically. The obtained data showed an increase of the relative abundances of IgG1 G0F and IgG2 G0F glycoforms with age (Figure 5A and E) which was in accordance with literature.⁸⁻¹⁰ Glycoforms exhibiting two galactoses, in contrast, were lower at higher age, which was true for G2F species (Figure 5B and F) as well as for their sialylated counterparts, G2FS (Figure 5C

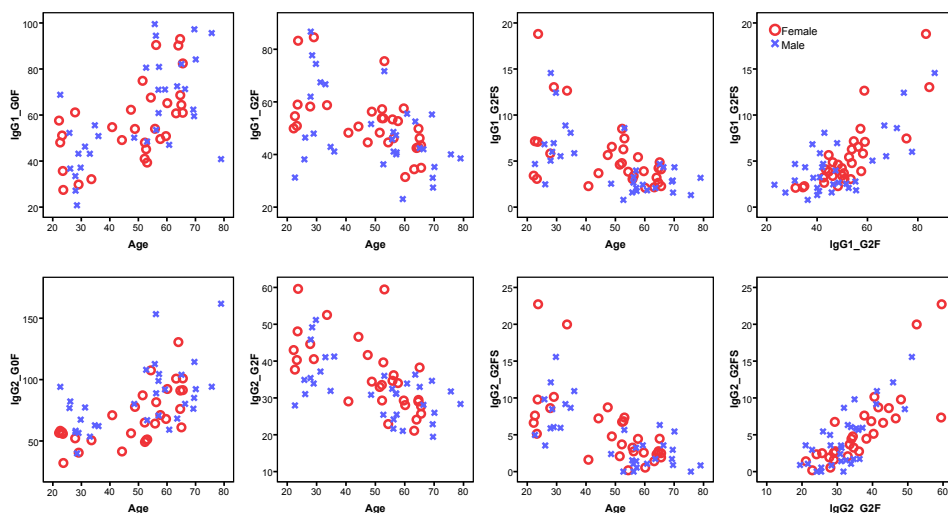


Figure 5. Changes in IgG glycosylation with age. IgG Fc N-glycan microheterogeneity was determined for a cohort of 62 individuals. The tryptic IgG glycopeptides were desalted and purified by Sepharose, spotted on a MALDI target plate, overlaid with CHCA, and analyzed by high resolution MALDI-FTICR-MS. Next to the age dependencies of G0F, G2F, and G2FS glycoforms of IgG1 and IgG2 (A-C and E-G), a correlation between G2F and G2FS was demonstrated (D and H).

and G). Moreover, two tailed Pearson correlation analysis revealed a strong correlation (p -values < 0.001) between galactosylation of the IgG1 and IgG2 Fc N-glycans and the level of sialylation, as exemplified for the glycoforms G2F and G2FS (Figure 5D and H). We additionally observed a significant correlation between the incidence of bisecting N-acetylglucosamine and age (p -values < 0.001) with increasing levels of bisecting N-acetylglucosamine at higher age (Figure S-4).

Discussion

We developed and applied a high-throughput method for IgG glycosylation profiling comprising sample preparation, mass spectrometric analysis, and data processing. The entire sample preparation was performed using the 96-well plate format. Human polyclonal IgGs were captured and purified from total plasma or serum using immobilized protein A, and cleaved with trypsin. The obtained glycopeptides were desalted by SPE and analyzed with MALDI-MS. In a first version of the protocol desalting and purification of the obtained glycopeptides was achieved with RP-SPE. Elution was performed with 18% ACN containing 0.1% TFA. Because such acidic conditions quickly lead to partial desialylation of glycopeptides, samples were dried after RP-SPE by vacuum centrifugation and dissolved in water, before they were used for sample spotting and MALDI-MS analysis. In order to shorten and simplify the sample preparation method, and to avoid possible sources of sample contamination, we replaced the RP-SPE by HILIC-SPE. Both HILIC materials tested (cellulose and Sepharose) provided very selective glycopeptide enrichment, which was in accordance to literature.^{102,188} Compared to the RP-SPE, the HILIC-SPE method was easier and faster, as eluates did not have to be dried by vacuum centrifugation and dissolved in water prior to mass spectrometric analysis. Elution from HILIC was performed with water making the samples directly available for analysis and long term storage. For both RP-SPE and HILIC-SPE some non-glycopeptide signals were observed which did not interfere with glycopeptide signals (Table S-1).

To our knowledge, the Fc N-glycosylation sites of human plasma IgG are fully occupied. Recombinant expression of antibodies, however, may result in only partial occupation of Fc N-glycosylation sites, as has been shown for single-chain Fv-Fc antibodies expressed in *Arabidopsis thaliana* seeds.¹⁸⁹ Therefore, monitoring of non-glycosylated variants of the tryptic Fc peptides carrying the N-glycosylation consensus sequence may be desirable in some cases. Notably, implementation of this analysis into the presented workflow is not compatible with HILIC-SPE, as non-glycosylated peptides will be lost during this purification step. RP-SPE, however, should be suitable for this purpose, as glycosylated as well as non-glycosylated versions of the tryptic Fc peptides will elute together.

For high-throughput IgG glycopeptide analysis we compared MALDI-TOF-MS with MALDI-FTICR-MS. When analyzed by positive-ion reflectron mode MALDI-TOF-MS

using delayed extraction, sialylated glycopeptides showed massive desialylation due to in-source and metastable decay. By contrast, detection of sialylated glycopeptides was allowed by linear mode MALDI-TOF-MS, since the lack of an extraction delay strongly reduced in-source decay, and metastable decay products formed after ion acceleration were not separated from their precursor ions. However, in this configuration the glycopeptides from polyclonal human IgG were poorly resolved, undermining the relative quantification of the different glycoforms. By using a 9.4 tesla FTICR-MS equipped with an intermediate pressure MALDI source, IgG Fc glycopeptides could be ionized without in-source decay of sialic acids allowing high mass resolution profiling of all IgG Fc N-glycopeptides. Notably, also other MALDI-MS systems with intermediate pressure sources such as MALDI-quadrupole-TOF instruments are suitable for profiling of IgG Fc glycopeptides including sialylated IgG glycopeptides. Analysis of the IgG glycopeptide samples by MALDI ionization on a SYNAPT™ High Definition Mass Spectrometry™ system (Waters) gave glycopeptide profiles which were comparable to those obtained by MALDI-FTICR-MS (data not shown).

With MALDI-FTICR-MS DHB and CHCA matrix both provided very robust and reproducible IgG glycopeptide profiles of similar intensities, which were stable over a wide laser power range. Notably, sialylated glycopeptides were registered with both matrices. The DHB preparations provided higher relative intensities of sialylated glycopeptides than observed with CHCA, possibly as a result of the “colder” character of DHB^{190,191} or by differences in ionization efficiencies. This indicated that in-source and metastable fragmentation were effectively reduced due to a combination of collisional cooling and low acceleration voltages applied in the intermediate pressure MALDI source. To our knowledge this is the first time that IgG glycopeptide analysis with detection of sialylated species is reported with CHCA. This mass spectrometric method offers the possibility to use stainless steel MALDI target plates, which are cheaper, more robust and less prone to contamination. Furthermore, the big spots with homogeneous, microcrystalline sample preparation allow multiple repeated analyses of the same sample.

The entire method comprising IgG capturing, tryptic cleavage, desalting by HILIC-SPE with Sepharose, MALDI-FTICR-MS using a polished stainless steel plate and sample overlay with CHCA, and automatic spectra processing was validated. This showed an interday variability below 10%, which was mainly due to the mass spectrometric measurement and was similar to the results obtained with LC-MS.⁴ Compared to LC-MS, however, MALDI-FTICR-MS analysis times are much shorter, and our method allows high-resolution IgG Fc glycosylation profiling of 384 samples in less than 36 hours, of which only roughly 18% account for the MALDI-FTICR-MS measuring time.

Here, we applied the method for analyzing polyclonal human IgGs, which results in some ambiguities in peak assignment due to structural isomers, i.e., non-fucosylated glycopeptides of one IgG subclass showing exactly the same molecular composition as

fucosylated glycopeptides of another IgG subclass (Table 1). This effect will not occur when the method is applied for the analysis of bioengineered monoclonal therapeutic antibodies, for which unambiguous assignment of molecular compositions of Fc N-glycopeptides will be possible.

IgG Fc N-glycans are known to show a low galactosylation in many diseases.¹⁴⁷ Moreover, IgG glycosylation features in healthy individuals reflect age, gender, and pregnancy.^{4,8-10,13,14} It was found that the percentage of agalactosylated IgG decreased during pregnancy and increased after delivery to values similar to those prior to conception.¹⁴ Moreover, IgG galactosylation has been found to be high at age 25 and is decreasing at higher age.⁸ Here, the developed high-throughput MALDI-FTICR-MS method was successfully applied to profile IgG Fc glycosylation of a group of 62 individuals. From these data, we were able to confirm the previous described age dependency of IgG galactosylation.⁸⁻¹⁰ We additionally observed an increased level for the incidence of bisecting N-acetylglucosamine with aging (Figure S-4), as described by Yamada *et al.*⁹ We did, however, not observe the described plateau reached at an age of 50 years. Notably, next to the age dependency of galactosylation, we found a pronounced age-dependency of sialylation, with IgG Fc sialylation being lower in old individuals than in young individuals (Figure 5C and G). The G2F glycan structure represents the substrate of the sialyltransferase reaction which results in G2FS. Not surprisingly, our data revealed a strong and significant correlation between Fc N-glycan galactosylation (G2F) and sialylation (G2FS) for both IgG1 and IgG2 (Figure 5D and H).

The IgG glycosylation analysis method described in this paper can handle large sample collections and reveals glycosylation features such as galactosylation, sialylation, fucosylation, and the incidence of bisecting N-acetylglucosamine in an IgG subclass-specific manner. The method is suitable for revealing IgG glycosylation features in large clinical cohorts and population studies. Moreover, it can be applied to analyze the Fc glycosylation of recombinantly produced IgG.

Acknowledgements

The authors thank Rudi G.J. Westendorp and Anton J.M. de Craen from the Department of Gerontology and Geriatrics of the Leiden University Medical Center and Jan J.G.M. Verschuuren, Erik H. Niks and Maarten J. Titulaer from the Neurology Department of the Leiden University Medical Center for kindly providing the human samples. Maurice H.J. Selman thanks Hoffmann la Roche for financial support.

Supporting information

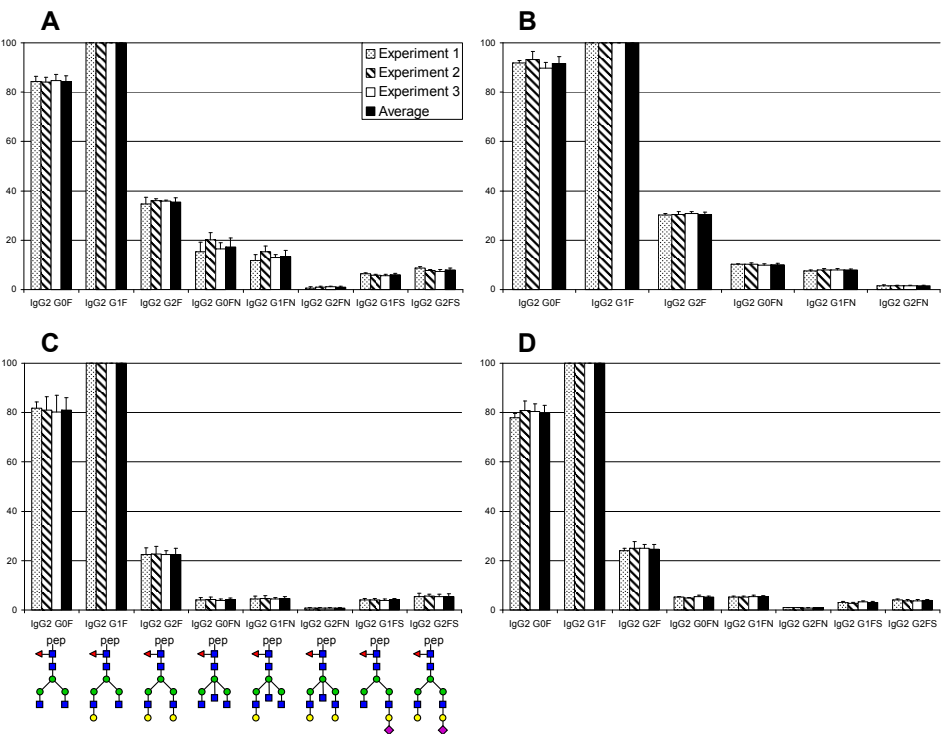


Figure S-1. Repeatability of 96-well RP-SPE for desalting and purification of tryptic IgG2 Fc glycopeptides. Analysis was performed by linear mode MALDI-TOF-MS with DHB (A), reflectron mode MALDI-TOF-MS using CHCA (B), and MALDI-FTICR-MS using DHB (C) and CHCA (D). Relative intensities and STDs were determined from three independent experiments comprising eight replicates each. Glycan code as in Figure 1 of Chapter 1.

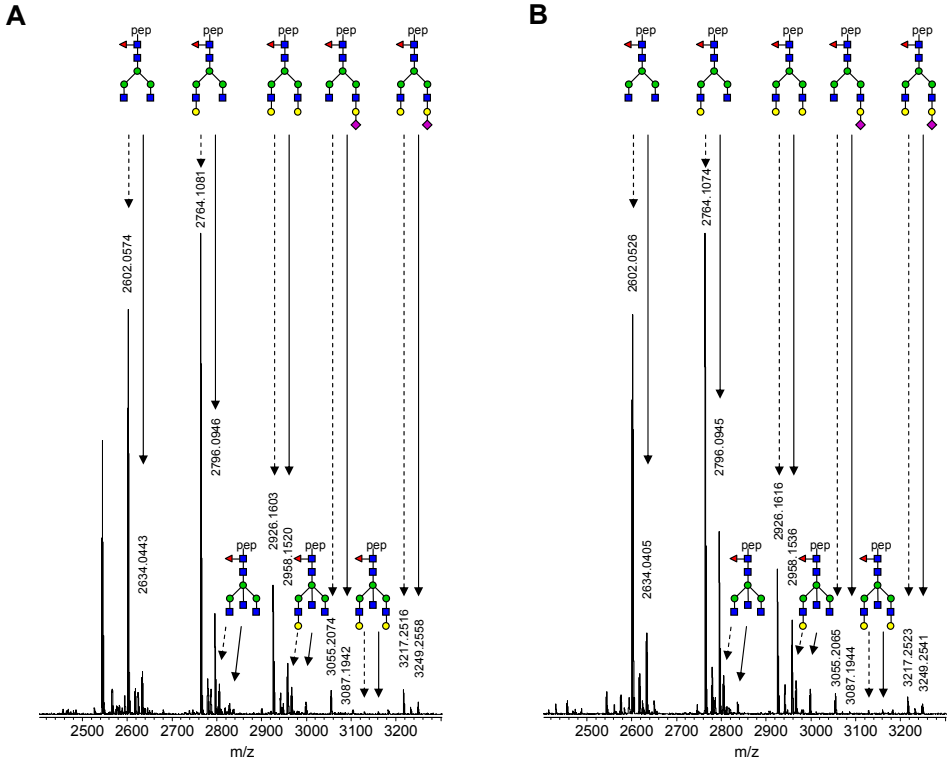
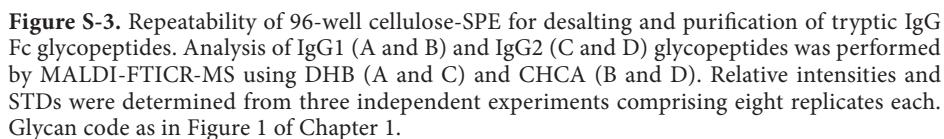


Figure S-2. MALDI-FTICR-MS analysis of cellulose-SPE purified and desalted tryptic IgG glycopeptides using DHB (A) and CHCA (B) as matrix substance. Continued arrows, IgG1 glycopeptides; dashed arrows, IgG2 glycopeptides; glycan code as in Figure 1 of Chapter 1.



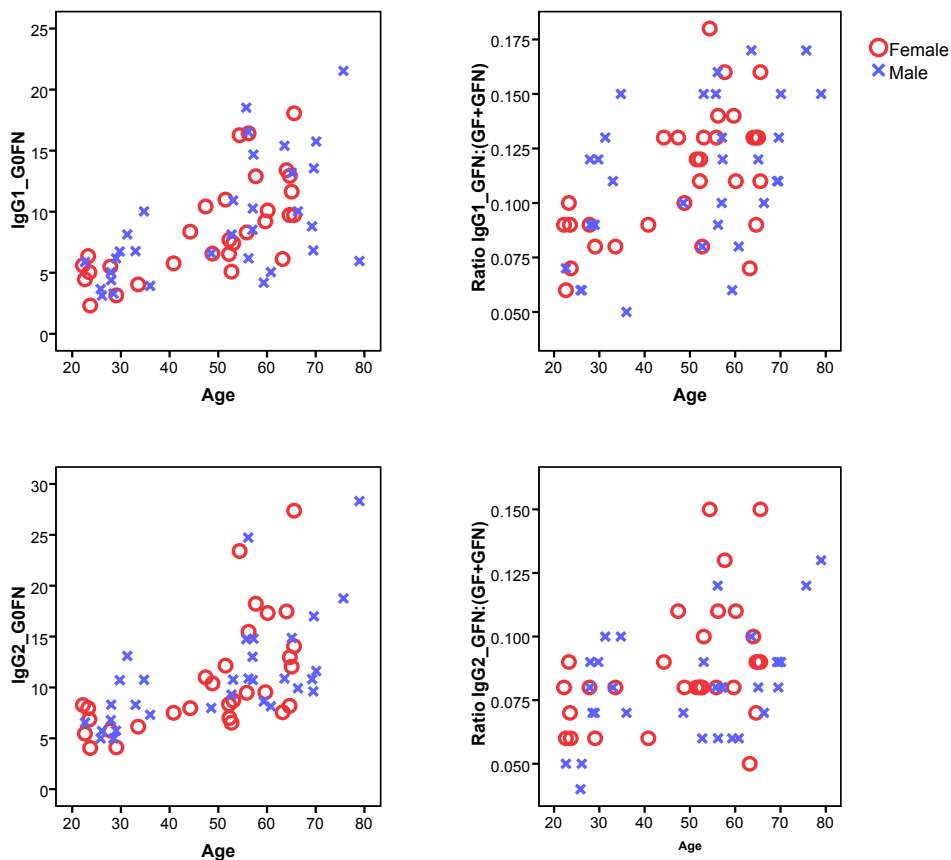


Figure S-4. Age-related changes in the level of bisecting N-acetylglucosamine for IgG1 (A and B) and IgG2 (C and D). IgG Fc N-glycan microheterogeneity was determined for a cohort of 62 individuals. The tryptic IgG glycopeptides were desalted and purified by Sepharose, spotted on a MALDI target plate, overlaid with CHCA, and analyzed by high resolution MALDI-FTICR-MS. The incidence of N-acetylglucosamine was determined with the formula $(G0FN+G1FN+G2FN)/(G0F+G1F+G2F+G0FN+G1FN+G2FN)$ and showed significant correlation with age for both IgG1 (B) and IgG2 (D).

Table S-1. Most frequently observed contamination peaks in MALDI-FTICR-MS IgG glycopeptide profiling of RP and HILIC-SPE preparations.

C18 _{end} SPE [M+H] ⁺	Cellulose and Sepharose SPE [M+H] ⁺
1920.9448	2043.0404
2135.9678	2135.9678
2157.9527	2157.9527
2459.0789	2173.9344
3084.3511	2195.9154
3246.4001	2548.1363
3278.3723	3619.6834
3408.4567	
3440.4059	

MALDI-TOF-MS analysis of sialylated glycans and glycopeptides using 4-chloro- α - cyanocinnamic acid matrix

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Proteomics, 2012, accepted.

Abstract

For MALDI analysis of glycans and glycopeptides the choice of matrix is crucial in minimizing desialylation by mass spectrometric in-source and metastable decay. Here we evaluated the potential of 4-chloro- α -cyanocinnamic acid (Cl-CCA) for MALDI-TOF-MS analysis of labile sialylated tryptic N-glycopeptides and released N- and O-glycans. Similar to 2,5-dihydroxybenzoic acid (DHB), but in contrast to α -cyano-4-hydroxycinnamic acid (CHCA), the Cl-CCA matrix allowed the analysis of sialylated N-glycans and glycopeptides in negative ion mode MALDI-TOF-MS. Dried droplet preparations of Cl-CCA provided microcrystals with a homogeneous spatial distribution and high shot-to-shot repeatability similar to CHCA, which simplified the automatic measurement and improved the resolution and mass accuracy. Interestingly, reflectron-positive ion mode analysis of 1-phenyl-3-methyl-5-pyrazolone (PMP) labeled O-glycans with Cl-CCA revealed more complete profiles than with DHB and CHCA. In conclusion, we clearly demonstrate the high potential of this rationally designed matrix for glycomics and glycoproteomics.

Introduction

During the last two decades MALDI-MS and ESI-MS have become the major soft ionization techniques widely applied for the analysis of a broad range of biomolecules. MALDI is most often combined with time-of-flight (TOF) mass analyzers and is particularly fast and easy to use. The development of faster instruments with higher mass accuracy and sensitivity, the implementation of new lasers, the advent of advanced TOF/TOF instruments as well as the introduction of new MALDI matrices have improved the technique and widened its application range. Since over two decades, 2,5-dihydroxybenzoic acid (DHB) and α -cyano-4-hydroxycinnamic acid (CHCA) are the 'gold standard' matrices for the MALDI-TOF-MS analysis of peptides, glycopeptides and glycans.^{191,192} Other matrices frequently applied include sinapinic acid, 3-hydroxypicolinic acid, 2,6-dihydroxyacetophenone and 2,4,6-trihydroxyacetophenone (THAP).^{191,192}

MALDI-TOF-MS is a very broadly applied method for the analysis of glycans and glycopeptides.^{148,191,192} However, when labile substituents such as sialic acids are present, the analysis can be complicated as these substituents may be lost by in-source and/or metastable decay. When analyzing glycans, sialic acids can be stabilized by methylation of carboxylic acid residues, or by permethylation.¹⁹³⁻¹⁹⁵ In case no such stabilization measures are undertaken, the degree of desialylation is strongly influenced by the chosen MALDI matrix. For example, CHCA as a hot matrix tends to lead to an almost complete loss of sialic acids, while DHB which is a colder matrix is less prone to sialic acids loss and, particularly in combination with negative ion mode and/or linear mode MALDI-TOF-MS, allows the analysis of sialylated glycans and glycopeptides without derivatization of the carboxylic acid groups.^{94,148,192}

In MALDI sample preparation, DHB tends to form large needle-shaped crystals that originate at the periphery of the target spot and project towards the center.¹⁹¹ Crystallization of released N-glycans with DHB (with or without sodium) generally results in more homogeneous microcrystals in the center of the spot whereas glycopeptides when subjected to identical crystallization conditions are found in the large crystals in the periphery¹⁹¹ and are detected preferably in protonated (positive ion mode) or deprotonated form (negative ion mode). The macrocrystalline nature of DHB often results in sample spot areas where certain analytes are highly concentrated (sweet spots), and in a very large spatial heterogeneity of mass spectra obtained from one sample spot.¹⁹⁶⁻¹⁹⁸ Moreover, MALDI-TOF-MS of macrocrystalline sample preparations on linear MALDI-TOF instruments (not on orthogonal MALDI-TOF instruments) is known to result in mass spectra of rather low resolution and mass accuracy. Recrystallization of sample spots using ethanol has been reported as a suitable mean to obtain a microcrystalline, more homogeneous glycan sample preparation with DHB,⁸⁵ but this may lead to signal suppression. No similar procedure, however, has been established for glycopeptides.

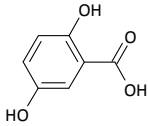
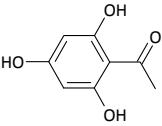
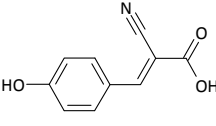
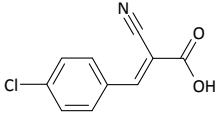
The large crystals obtained in sample preparations with DHB and the resulting large shot-to-shot variation, low mass accuracy and resolution made us search for an alternative, microcrystalline matrix that is compatible with the analysis of glycans and glycopeptides bearing labile substituents such as sialic acids. Here, we describe the use of 4-chloro- α -cyanocinnamic acid (Cl-CCA, systematic name 3-(4-chloro-phenyl)-2-cyano-acrylic acid) for the analysis of glycans and glycopeptides in linear- and reflectron-negative ion mode MALDI-TOF-MS. Cl-CCA has recently been introduced as a rationally designed MALDI matrix for proteomics¹⁹⁹ and has been found to be suitable for the analysis of labile molecules.²⁰⁰ We compared Cl-CCA with conventionally used matrices, i.e. DHB, THAP and CHCA (see Scheme 1 in supporting information for the chemical structures) for the analysis of sialylated tryptic glycopeptides and released N- and O-glycans. We found Cl-CCA to be a colder matrix than CHCA, more similar to DHB,²⁰⁰ allowing the detection of sialylated species. Moreover, Cl-CCA preparations resulted in microcrystalline sample spots for glycans and glycopeptides, making this matrix highly suitable for automated measurement without compromising resolution and mass accuracy.

Experimental section

IgG purification. IgGs (ca. 20 μ g) were affinity captured from total human plasma (2 μ l) as described previously.¹⁴⁸

Reduction and alkylation. Bovine fetuin or human apo-transferrin (1 mg; Sigma-Aldrich, Steinheim, Germany) was dissolved in 200 μ l 50 mM ammonium bicarbonate buffer_(aq) (Fluka) containing 10 mM dithiothreitol_(aq) (Sigma-Aldrich) and reduced at 60°C for 40 min. Cysteine alkylation was achieved by addition of 30 μ l 100 mM iodoacetamide_(aq) (Sigma-Aldrich) dissolved in 50 mM ammonium bicarbonate buffer followed by 30 min incubation at RT in the dark. The alkylation reaction was stopped by putting the sample under a fluorescent lamp (gas discharge lamp) for 30 min.

Trypsin digestion. Purified plasma IgGs (ca. 20 μ g) were digested with trypsin as described previously.¹⁴⁸ For tryptic digestion of bovine fetuin or human apo-transferrin,

			
2,5-dihydroxybenzoic acid (DHB)	2,4,6-trihydroxyacetophenone (THAP)	α -cyano-4-hydroxycinnamic acid (CHCA)	4-chloro- α -cyanocinnamic acid (Cl-CCA)

Scheme 1. Chemical structures of the MALDI matrices DHB, THAP, CHCA and Cl-CCA.

92 μ l (0.4 mg) of the reduced and alkylated glycoprotein sample was digested overnight at 37°C with lyophilized sequencing grade modified trypsin (Promega, Madison, WI) in a 1:20 (w/w) enzyme to substrate ratio.

In solution N-Glycosidase F release. N-glycans were released from 60 μ l (0.3 mg) of the reduced and alkylated tryptic digests of bovine fetuin or human apo-transferrin by an overnight incubation at 37°C with 10 mU PNGase F (Roche Applied Science, Mannheim, Germany).

HILIC SPE purification. 96-Well plate format cellulose hydrophilic interaction liquid chromatography solid phase extraction (HILIC SPE) of tryptic IgG glycopeptides (ca. 20 μ g IgG) was performed as described previously.¹⁴⁸ Cotton HILIC micro-SPE was performed as described previously²⁰¹ with minor modifications. Briefly, 1 μ l of tryptic glycopeptides (corresponding to ca. 4.3 μ g protein) or 2 μ l of PNGase F released N-glycans (corresponding to ca. 8.6 μ g protein) from bovine fetuin and human apo-transferrin were brought to 83% acetonitrile (ACN) and adsorbed on a cotton HILIC microSPE conditioned with 83% ACN by aspirating and dispensing the sample twenty times. The retained analytes were washed 3 times with 10 μ l of 83% ACN containing 0.1% trifluoroacetic acid and eluted with 10 μ l of water.

Glycopeptide and glycan spotting. Glycopeptides (1 μ l) derived from 220 ng IgG, 430 ng fetuin or transferrin, or released glycans (1 μ l) derived from 860 ng fetuin or transferrin were spotted either onto an MTP AnchorChip 600/384 or polished stainless steel MALDI target plate (Daltonics, Bremen, Germany) and allowed to dry by air at RT. To the dry sample spots on the AnchorChip 1 μ l of DHB (5 mg/ml 50% ACN_(aq); purity not defined; Bruker Daltonics, Bremen, Germany),¹⁴⁸ THAP (1 mg/ml 50% ACN_(aq); purity not defined; Bruker Daltonics) or THAP/diammonium citrate (1 mg THAP and 1mM diammonium citrate in 1 ml 50% ACN_(aq))²⁰² was added. To the dry sample spots on the polished steel MALDI plate 1 μ l of CHCA (5 mg/ml 50% ACN_(aq); purity not defined; Bruker Daltonics),¹⁴⁸ or Cl-CCA (5 mg/ml 70% ACN_(aq); 95% purity; Bionet Research, Camelford, Cornwall, UK) was applied. Matrix specific spotting and drying conditions were chosen close or identical to previously described effective procedures.^{148,199,202}

N-glycan permethylation. Released N-glycans (derived from 1 mg fetuin or transferrin) were applied to a reversed-phase solid phase extraction (SPE) cartridge (500 mg Bakerbond end capped C18; Mallinckrodt Baker, Deventer, NL). The flow-through and wash (5 ml water) were applied to a carbon cartridge (150 mg Carbograph; Grace, Lokeren, BE). The retained glycans were washed with water (5 ml), eluted with 25% aqueous ACN (5 ml) and dried by freeze-drying. The purified and desalted glycans were permethylated as described previously²⁰³ and reconstituted in 20 μ l ACN. Aliquots (1 μ l) were spotted onto a polished steel MALDI plate, dried by air at RT, overlaid with DHB (1 μ l; 10 mg/ml 50% ACN_(aq)) and analyzed by reflectron-positive ion mode MALDI-TOF-MS as described below.

Preparation of PMP-labeled Mucin O-glycans. O-glycans were released from mucin of bovine submaxillary glands (Sigma-Aldrich) and labeled with 1-phenyl-3-methyl-5-pyrazolone (PMP) as described previously.²⁰⁴ The yields of the reactions were checked by nanoRP-LC-MS²⁰⁴ to ensure sample suitability for O-glycan profiling. From the aqueous PMP-labeled O-glycan sample 10 μ l aliquots (corresponding to approximately 500 ng of mucin starting material) were purified with a RP C18 ziptip according to the manufacturers protocol (Millipore), directly eluted with 2 μ l DHB (5 mg/ml 50% ACN_(aq)) or 3 μ l Cl-CCA (5 mg/ml 50% ACN_(aq); Bionet Research) onto a MALDI target plate and analyzed by reflectron-positive ion mode MALDI-TOF-MS as described below.

MALDI-TOF-MS analysis. Samples were analyzed on an Ultraflex II MALDI-TOF/TOF-MS (Bruker Daltonics) in reflectron-positive, reflectron-negative, and linear-negative ion mode. Calibration of the MALDI-TOF-MS spectra was performed with standard peptide mixtures from Bruker Daltonics. The samples were irradiated by a smartbeam™ 200 Hz solid-state laser. For reflectron mode analysis a 160 ns delayed extraction was applied after which the ions were accelerated with 25 kV, while the ions were accelerated immediately without delayed extraction in the linear mode. The ions were recorded between m/z 1300 and 4600 (N-glycans and IgG glycopeptides), m/z 1300 and 8000 (fetuin and transferrin glycopeptides), m/z 960 and 4500 (permethylated fetuin and transferrin glycans) or m/z 440 and 3700 (O-glycans). The ions below m/z 300 (O-glycans), 500 (N-glycans and N-glycopeptides) or 800 (permethylated N-glycans) were suppressed. Each mass spectrum was generated by summing the ions of 1000 (O-glycans), 2000 (N-glycans and N-glycopeptides) or 4000 (permethylated N-glycans) laser shots. The laser intensity was optimized to give the best signal-to-noise (S/N) ratio for each sample. Automatic MALDI-TOF-MS analysis, was performed as describe previously.¹⁴⁸ All data processing and evaluation were performed with FlexAnalysis Software (Bruker Daltonics) and Microsoft Excel, respectively. Relative intensities of the Fc N-glycopeptide species derived from IgG1 and IgG2 (8 glycoforms each) were obtained by normalization to the total IgG subclass specific glycopeptide intensities.

Limit of detection. To evaluate the limit of detection (LOD) of MALDI-TOF-MS with DHB and Cl-CCA we prepared a dilution series ranging from 0.001 to 50 ng/ μ l of a tryptic digest of a monoclonal antibody. In addition, a maltoheptaose dilution series was prepared ranging in concentration from 0.05 to 50 ng/ μ l. From each dilution 1 μ l was spotted onto an AnchorChip target plate and dried by air. To the dried sample spots 1 μ l of DHB (5 mg/ml 50% ACN_(aq)),¹⁴⁸ or 1 μ l of Cl-CCA (1 mg/ml 70% ACN_(aq)) was applied. Matrix specific spotting and drying conditions were chosen identical or close to previously described effective procedures.^{148,199} Spots were analyzed in reflectron-positive and reflectron-negative ion mode.

In the Fc portion of each antibody two N-glycans are located, which may vary in glycan composition. Calculation of the LOD for the Fc-derived tryptic glycopeptides of a monoclonal antibody was performed as follows:

$$\text{LOD (mol)} = \frac{\text{Lowest measurable antibody amount on spot (g)} * 2 (\text{Number of Fc N-glycans})}{150\,000 (\text{g/mol; molecular mass antibody}) * \text{Number of observed glycoforms}}$$

Results

MALDI-TOF-MS of IgG glycopeptides. Human polyclonal IgGs purified by protein A affinity chromatography from plasma of a healthy volunteer were digested by trypsin and subsequently desalted and purified using cellulose HILIC SPE. Purified IgG glycopeptides were spotted onto a MALDI target plate and co-crystallized with matrix (DHB, CHCA, THAP, or Cl-CCA). Initial Cl-CCA pilot experiments were performed with commercially obtained matrix and matrix kindly provided by the group of Prof. Dr. M. Karas. The matrix solution of the commercially obtained Cl-CCA was prepared in 50, 60 and 70% ACN at 5 mg/ml, while the matrix solution of the provided Cl-CCA was prepared as described previously.¹⁹⁹ Matrices obtained from the two different sources gave very similar results, with the best S/N ratio being obtained for the 70% ACN preparation (data not shown).

DHB preparations showed very dense, thick crystals covering the AnchorChip spot (Figure 1A). For THAP matrix dried by vacuum fairly homogeneous crystals were observed. When dried at atmospheric pressure, however, THAP preparations were heterogeneous with long crystals more similar to DHB. By contrast, CHCA and Cl-CCA preparations showed small crystals with a homogeneous spatial distribution. The Cl-CCA preparations occasionally showed a white crust ring of matrix crystals with very small, white crystals within the ring (Figure 1B).

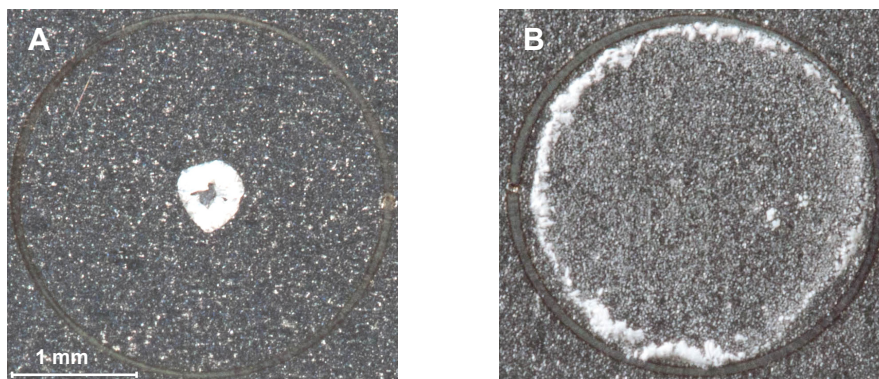
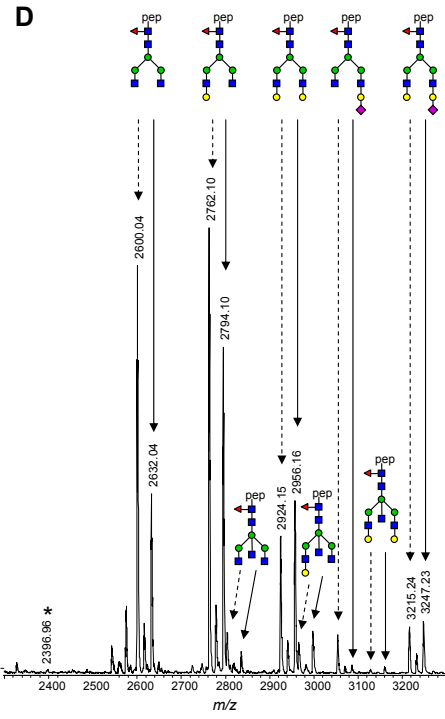
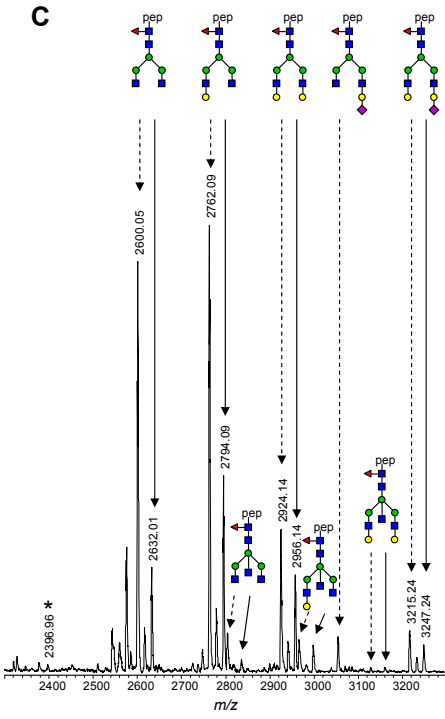
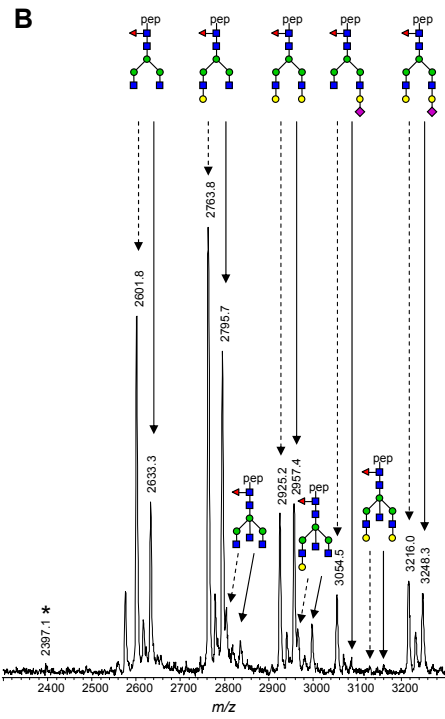
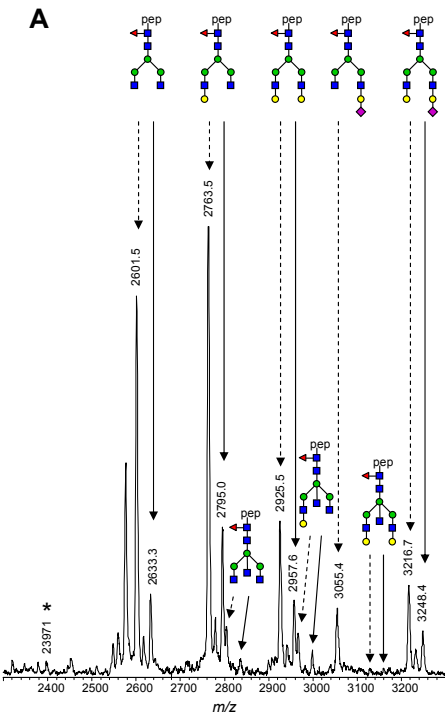


Figure 1. Magnified image of tryptic IgG glycopeptides co-crystallized with DHB onto an AnchorChip MALDI plate (A) or Cl-CCA onto a polished steel MALDI plate (B). The spot formation shows a heterogeneous macrocrystalline DHB spot and a homogeneous microcrystalline Cl-CCA matrix spot. Notably, Cl-CCA matrix is producing a thin white crust ring around the matrix spot.

Analysis of the ring of such spots revealed similar glycopeptide profiles with a slightly lower S/N ratio and resolution compared to profiles obtained in the center. This, however, did not affect the automatic MALDI-TOF-MS measurements as the ring was not sampled with the used random walk laser movement. For Cl-CCA preparations, intense IgG glycopeptide signals were obtained over the entire sample spot. Spots older than 2 weeks and stored on the bench still provided intense glycopeptide signals. When stored under vacuum in the MALDI source during 8 h no decrease in IgG glycopeptide intensity was observed (data not shown). Sample spots stored for over 24 h in the MALDI source, however, allowed registration of IgG glycopeptides only in the larger not yet fully sublimated crystals of ring. The Cl-CCA and DHB preparations required a similar laser power for threshold signals, which were ca. 2-3% higher than for CHCA or 5-6% lower than for THAP (with or without diammonium citrate) preparations.

Independent of the measurement mode and MALDI matrix the most abundant signals were obtained for the glycopeptides of IgG2 followed by IgG1 and IgG4 (Figure 2 and Figure S-1). The observed profiles closely resembled the reversed phase nanoLC-ESI-MS IgG glycopeptide profiles obtained for the same sample. The assignments of the IgG glycopeptides were confirmed by MALDI-TOF/TOF-MS/MS and nanoLC-ESI-ion trap-MS/MS analysis of the IgG1 and IgG2 G0F species providing IgG subclass specific peptide sequence information (data not shown). In reflectron-positive ion mode MALDI-TOF-MS using CHCA (Figure S-1A) and Cl-CCA (Figure S-1B) matrix, IgG glycopeptide spectra with a good S/N ratio were obtained showing almost exclusively neutral glycoforms with only very minor signals for the sialylated species. Similarly, sialylated glycoforms were of very low relative abundance when using CHCA matrix in reflectron-negative ion mode (Figure S-1C). By contrast, DHB (Figure 2A and C) and Cl-CCA (Figure 2B and D) allowed detection of sialylated glycopeptides next to the neutral glycoforms in the linear-negative (Figure 2A and B) and reflectron-negative ion mode (Figure 2C and D). Interestingly, an in-source decay product at m/z 2396.96 ($[M-H]^+$) was observed at lower relative intensity with Cl-CCA as compared to DHB. This glycopeptides species is formed when the linkage breaks between an antenna GlcNAc and a core mannose of the IgG2 G0F or G1F species. The absence of this glycan species from the original IgG glycopeptide sample was confirmed by nano-reversed phase liquid chromatography electrospray ionization MS, indicating that the observation of this glycopeptide ion in MALDI-TOF-MS is related to in-source decay (data not shown). With the THAP matrix only low quality IgG glycopeptide profile

Figure 2. MALDI-TOF-MS spectra of tryptic IgG Fc N-glycopeptides co-crystallized with DHB (A, C) or Cl-CCA (B, D) and measured in linear-negative (A, B) and reflectron-negative ion mode (C, D). Profiling of IgG Fc N-glycopeptides was performed after 96-well format cellulose-HILIC purification. Dashed arrows, IgG2 glycopeptides; solid arrows, IgG1 glycopeptides; *, in-source fragment; glycan code as in Figure 1 of Chapter 1.



spectra with high levels of alkali salt adducts were obtained in reflectron-positive (Figure S-2A) and reflectron-negative (Figure S-2B) ion mode. While the presence of diammonium citrate in the THAP matrix significantly reduced the observed alkali salt adduct levels and improved the spectra quality (Figure S-2C and D), the observed S/N ratio and resolution were consistently lower compared to the other matrices. In an effort to improve the IgG glycopeptide spectra with THAP/diammonium citrate the matrix was dried underneath the pierced cap similar to the procedure applied with DHB. While this improved the observed IgG glycopeptide signals, the S/N ratio of ca. 45 remained much lower than for DHB and Cl-CCA (Figure 3).

To evaluate the performance of DHB and Cl-CCA for glycopeptide profiling by negative ion mode MALDI-TOF-MS, the purified IgG Fc N-glycopeptide sample was spotted eight times on an AnchorChip and polished steel target plate and overlaid with DHB or Cl-CCA, respectively. For both matrices a single spot was measured eight times in linear- and reflectron-negative ion mode after which one spectrum was acquired

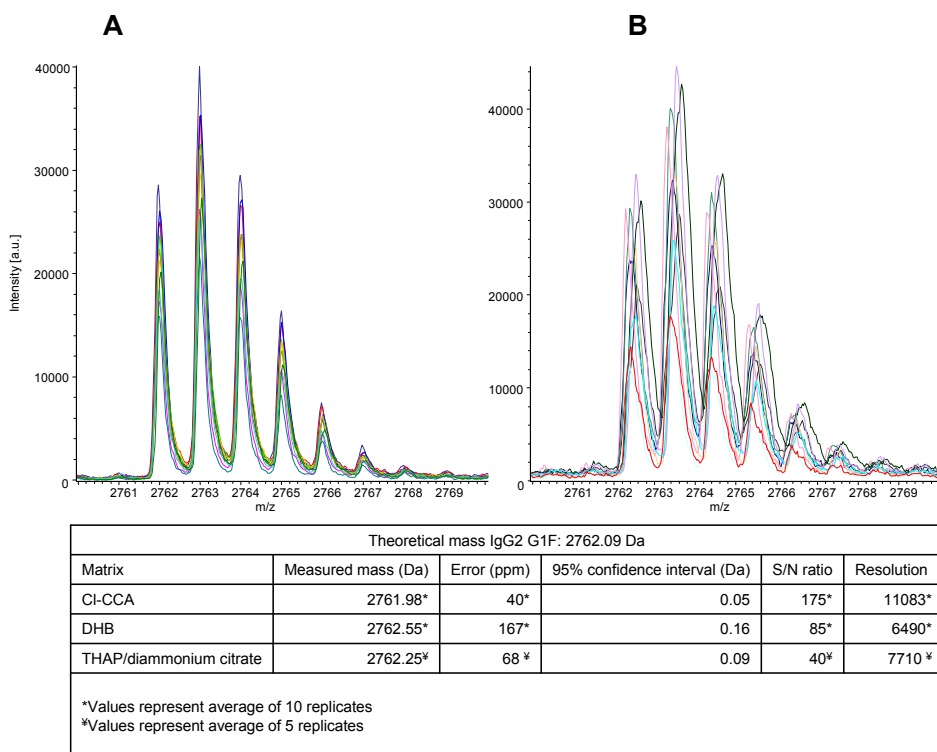


Figure 3. Overlay of the IgG2 G1F species from 10 reflectron-negative ion mode MALDI-TOF-MS spectra. Tryptic IgG Fc N-glycopeptide preparations were measured automatically on a polished steel target plate with Cl-CCA (A) or an AnchorChip plate with DHB (B) or THAP/diammonium citrate.

for each of the remaining replicate spots. Mass spectra were processed automatically which involved internal calibration, baseline subtraction, and peak picking. Within the spot and between the spots the relative standard deviations of the normalized peak heights for the six major glycoforms of IgG1 and IgG2 remained below 6 and 8% for the reflectron- and linear-negative ion mode measurements, respectively.

Next, eight replicates of the above described IgG Fc N-glycopeptide sample were prepared, spotted onto an AnchorChip (DHB) or polished steel target plate (Cl-CCA), and analyzed by reflectron- and linear-negative ion mode MALDI-TOF-MS. This experiment was performed three times each on different days. Within the three experiments, the intraday and interday variability was determined for IgG1 (Figure S-3) and IgG2 (Figure S-4). Again the relative standard deviations for the six major glycoforms of IgG1 and IgG2 in the reflectron- and linear-negative ion mode measurements remained below 6 and 8%, respectively. The homogeneous spatial distribution of the Cl-CCA microcrystals provided a high shot-to-shot repeatability, which simplified the automatic measurement and improved the resolution and mass accuracy of the MALDI-TOF-MS measurements as demonstrated by comparing replicate spectra (Figure 3).

To evaluate the LOD of MALDI-TOF-MS with DHB and Cl-CCA a dilution series of a tryptic digest of a monoclonal antibody was prepared. For both matrices, the dilutions were spotted onto an AnchorChip MALDI target plate, cocrystallized with DHB or Cl-CCA, and analyzed in the reflectron-positive and reflectron-negative ion mode. The lowest measurable monoclonal antibody amount in the reflectron-negative ion mode with DHB or Cl-CCA was 0.1 and 0.05 ng on spot, respectively. In the reflectron-positive ion mode with DHB or Cl-CCA the lowest measurable monoclonal antibody amount was 0.05 and 0.01 ng on spot, respectively. Upon reflectron mode (negative and positive) analysis with DHB or Cl-CCA of the lowest measurable monoclonal antibody amount three different glycoforms (S/N ratio ≥ 3) were observed. The LOD of the reflectron-negative ion mode measurements with DHB and Cl-CCA was, thereof, determined to be approximately 444 and 222 amol of glycopeptides on spot, respectively. In reflectron-positive ion mode the LOD was found to be 222 and 44 amol glycopeptides on spot for DHB and Cl-CCA, respectively. In addition, we analyzed a maltoheptaose dilution series in reflectron-positive and reflectron-negative ion mode with DHB and Cl-CCA. In the positive ion mode both matrices provided a similar LOD of approximately 10 fmol on spot, while in the negative ion mode no maltoheptaose signals could be obtained.

MALDI-TOF-MS of highly sialylated glycopeptides. In order to evaluate the suitability of the Cl-CCA matrix for negative ion mode MALDI-TOF-MS of glycopeptides with a high level of sialylation, we additionally prepared tryptic digests of bovine fetuin and human apo-transferrin, selectively purified the glycopeptides by cotton HILIC micro-SPE,²⁰¹ and spotted the sample directly onto an AnchorChip (DHB) and polished steel (Cl-CCA) MALDI target plate. MALDI-TOF-MS analysis was performed in the reflectron- and linear-negative ion mode. Fetuin glycopeptides

were in addition analyzed in the reflectron-positive ion mode. As expected, tryptic fetuin (Figure S-5) and transferrin (Figure S-6C and D) glycopeptides revealed large unfocused metastable peaks for highly sialylated structures in the reflectron mode analysis with both matrices. In linear-negative ion mode with DHB good signals were obtained for the di- and tri-antennary sialylated fetuin N-glycopeptides with the most abundant signals for the Leu₁₄₅-Arg₁₅₉ glycopeptide cluster (small peptide moiety), followed by the Arg₇₂-Arg₁₀₃ and Val₁₆₀-Arg₁₈₇ glycopeptide clusters (big and intermediate-size peptide moieties, respectively; Figure 4A). Linear-negative ion mode MALDI-TOF-MS of Cl-CCA overlaid fetuin glycopeptides revealed similar but slightly more intense profiles compared to DHB up to m/z 5000, while above m/z 5000 glycopeptide intensities were rather low (Figure 4B). Spectra of comparable quality were observed for human apo-transferrin overlaid with DHB (Figure S-6A) and

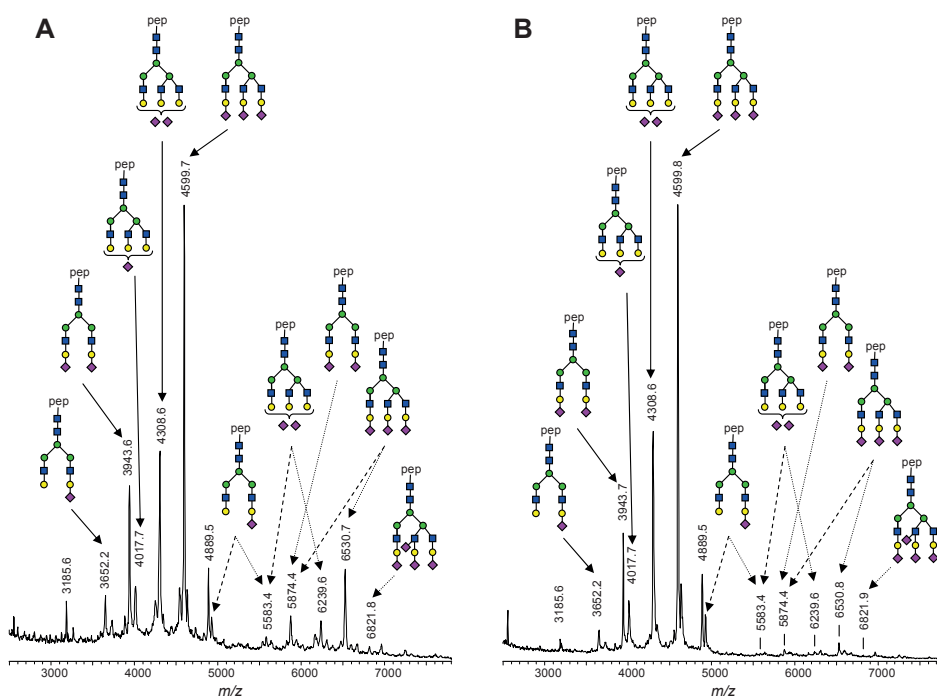


Figure 4. Linear-negative ion mode MALDI-TOF-MS spectra of tryptic bovine fetuin glycopeptides (SwissProt entry number: P12763). Tryptic glycopeptides were purified by cotton HILIC micro-SPE and measured on an AnchorChip target plate with DHB (A) and a polished steel target plate with Cl-CCA (B). Solid arrows, small molecular mass glycopeptide (L₁₄₅CPDCPLLAPLNSR₁₅₉); dashed arrows, intermediate molecular mass glycopeptide (V₁₆₀VHAVEVALATFNAESNGSYQLVEISR₁₈₇); dotted arrows, large molecular mass glycopeptide (R₇₂PTGEVYDIEIDTLETTCHVLDPTPLANCSVR₁₀₃); *, post source decay products; glycan code as in Figure 1 of Chapter 1.

Cl-CCA (Figure S-6B), revealing similar relative intensities for the large (Gln₆₂₂-Arg₆₄₂) and small (Cys₄₂₁-Lys₄₃₃) N-glycopeptides.

MALDI-TOF-MS of released N-glycans. The performance of the Cl-CCA matrix was also evaluated for MALDI-TOF-MS analysis of released N-glycans. To this end, N-glycans were released from bovine fetuin and human apo-transferrin with PNGase F, purified by cotton HILIC micro-SPE,²⁰¹ co-crystallized with DHB (AnchorChip), Cl-CCA (polished steel), THAP or THAP/diammonium citrate (AnchorChip) and analyzed by linear-negative ion mode MALDI-TOF-MS.

With DHB, highly sialylated fetuin N-glycans were detected with a good S/N ratio, showing predominantly singly charged deprotonated, sodiated and potassiated species (Figure 5A). In addition, low amounts of adducts with two sodium, two potassium or a sodium and potassium were observed for the tri- and tetra-sialylated N-glycans. Spectra of Cl-CCA overlaid fetuin N-glycans revealed lower relative intensities for the mono-sialylated fetuin glycopeptides compared to DHB, while similar relative intensities were observed for the multiple sialylated glycans with similar levels of alkali salt adducts (Figure 5B). Moreover, the relative abundance of partially sialylated species measured with Cl-CCA was lower compared to DHB. With Cl-CCA preparations very intense non-glycan related signals were observed at m/z 1991.1 ($[M-H]^-$, His₃₁₃-Lys₃₃₃) and 2119.3 ($[M-H]^-$, His₃₁₃-Gly₃₃₂) which were present only at low intensity with DHB preparations and were identified by MALDI-TOF/TOF-MS as fetuin peptides. THAP (Figure S-7A) and THAP/diammonium citrate (Figure S-7B) co-crystallized fetuin glycans revealed linear negative ion mode spectra of low quality for the released fetuin glycans which were dominated by alkali salt adducts. To improve the glycan spectra, the fetuin samples were recrystallized with 60% ethanol or 60% ethanol containing 1 mM sodium acetate, revealing similar spectra as obtained prior to the recrystallization (data not shown).

Analysis of Cl-CCA co-crystallized transferrin N-glycans (Figure S-8B) revealed spectra with N-glycan profiles and alkali salt adduct levels comparable to those observed with DHB (Figure S-8A) but with a lower S/N ratio. When analyzed in the linear- and reflectron-positive ion mode the Cl-CCA spots with released transferrin N-glycans revealed spectra with low intensity signals which predominantly originated from sodium adducts (data not shown). Transferrin glycans co-crystallized with THAP (Figure S-8C) or THAP/diammonium citrate (Figure S-8D) revealed similar linear-negative ion mode glycan profiles as obtained with DHB and Cl-CCA. While the spectra for transferrin N-glycans with DHB and Cl-CCA were dominated by deprotonated glycan species, spectra of transferrin N-glycans with THAP (with and without diammonium citrate) showed for a big part an exchange of acidic protons for sodium. As with the fetuin N-glycans, recrystallization of the transferrin N-glycans with 60% ethanol or 60% ethanol containing 1 mM sodium acetate resulted in similar spectra as observed prior to the recrystallization (data not shown).

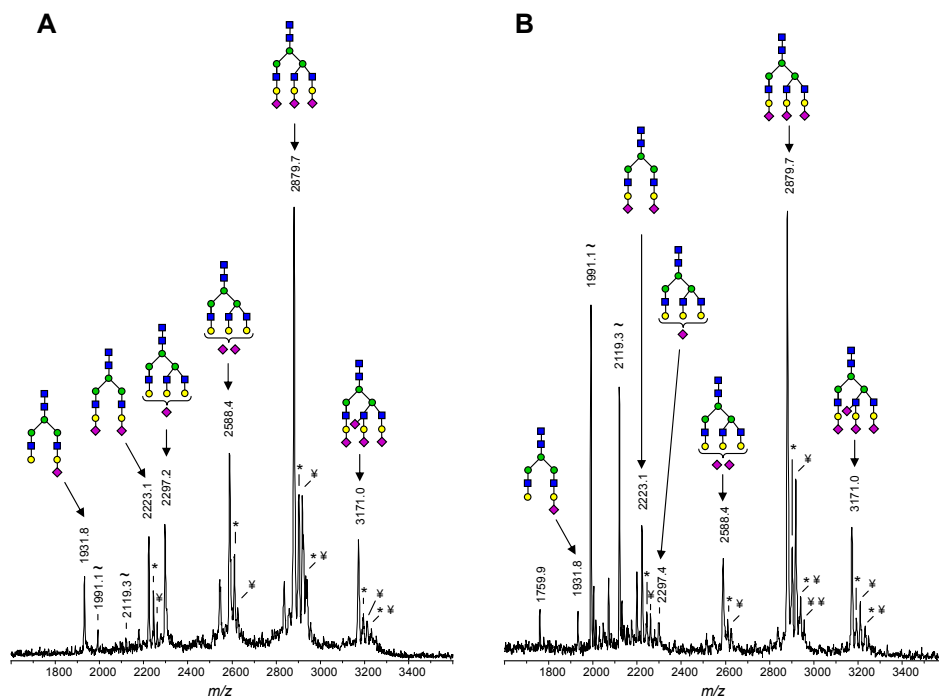


Figure 5. Linear-negative ion mode MALDI-TOF-MS spectra of PNGase F released bovine fetuin N-glycans. Released N-glycans were purified by cotton HILIC micro-SPE and co-crystallized with DHB on an AnchorChip (A) or Cl-CCA on a polished steel target plate (B) for analysis. ~, Fetuin peptide H₃₁₃ TFSGVASVESSSGEAFHVG K₃₃₃; *, sodium adduct; ‡, potassium adduct; glycan code as in Figure 1 of Chapter 1.

MALDI-TOF-MS of permethylated N-glycans. To evaluate the suitability of Cl-CCA for quantitative MALDI-TOF-MS analysis, the released fetuin and transferrin N-glycans were permethylated, co-crystallized with DHB and analyzed by reflectron-positive ion mode MALDI-TOF-MS. The most abundant fetuin N-glycan intensities (Figure S-9A) originated from the fully sialylated bi- and triantennary glycans, while only low signals were observed for the partially sialylated bi- and triantennary glycans. For transferrin (Figure S-9B) the most abundant signals originated from the fully sialylated biantennary glycans, while low signals were observed for the partially sialylated biantennary glycans and fully and partially sialylated triantennary glycans. Interestingly, the observed N-glycan profiles after permethylation were more similar to the negative ion mode MALDI-TOF-MS N-glycan profiles observed with Cl-CCA than to those observed with DHB.

Comparison of PMP-labeled O-glycan profiles using DHB and Cl-CCA. To evaluate the applicability of Cl-CCA for profiling small glycans, PMP-labeled mucin O-glycans

were co-crystallized with DHB (AnchorChip), CHCA, or Cl-CCA (polished steel) and analyzed by reflectron-negative and positive ion mode MALDI-TOF-MS. While in reflectron-negative ion mode we were unable to generate spectra of good quality for PMP-labeled O-glycans, reflectron-positive analysis with Cl-CCA provided spectra with a higher S/N ratio as compared to DHB (Figure S-10). No PMP-labeled O-glycans were observed with CHCA (data not shown). However, we were unable to detect the entire PMP-labeled mucin O-glycan set that has been described previously.^{204,205} As an alternative attempt to improve the mucin O-glycan coverage we co-crystallized the PMP-labeled O-glycans with Cl-CCA on an AnchorChip plate and repeated the analysis. More intense PMP-labeled O-glycan signals were obtained revealing two additional mucin O-glycans (m/z [1046.5+H]⁺ and [1062.6+H]⁺) corresponding to HexNAc₂NeuAc₁(PMP)₂ and HexNAc₂NeuGc₁(PMP)₂, respectively (HexNAc, N-acetylhexosamine; NeuAc, N-acetylneuraminic acid; NeuGc, N-glycolylneuraminic acid; Figure S-11).

Discussion

MALDI-TOF-MS analysis of sialylated glycoconjugates often suffers from massive desialylation due to in-source and metastable decay. Recently, the group of Prof. Dr. M. Karas introduced the rationally designed MALDI matrix Cl-CCA for proteomics,¹⁹⁹ which showed a high potential for the analysis of labile molecules.²⁰⁰ Compared to CHCA, Cl-CCA was found to be a colder matrix that allowed more efficient analyte protonation due to the lower proton affinity. Moreover, improved peptide sequence coverage, sensitivity, and detection of labile peptides has been reported.²⁰⁶ Here, we have evaluated the potential of Cl-CCA for the detection of labile sialylated glycans and glycopeptides. To this end tryptic glycopeptides (IgG, fetuin and transferrin), released N-glycans (fetuin and transferrin), and released O-glycans (bovine submaxillary gland mucin) were prepared and analyzed by MALDI-TOF-MS.

In a first attempt we compared tryptic IgG Fc N-glycopeptide MALDI-TOF-MS profiles obtained with different matrices (i.e. DHB, THAP, CHCA and Cl-CCA) and detection modes (i.e. reflectron-positive, reflectron-negative and linear-negative). The observed profiles closely resembled the reversed phase nanoLC-ESI-MS IgG glycopeptide profiles obtained for the same sample. Subclass specific peptide sequence information could be obtained by fragmentation of the G0F species of IgG1 and IgG2, which combined with literature knowledge of IgG N-glycosylation^{3,4,9-11,79,148} allowed structural assignment of the detected glycoforms with good confidence. While IgG1 is present at a higher concentration in the blood circulation than IgG2, the latter showed higher MS intensities possibly due to a more efficient ionization of the phenylalanine containing IgG2 glycopeptide compared to the tyrosine containing IgG1 glycopeptide. The slightly higher hydrophobicity of the peptide portion of the IgG2 glycopeptide may also promote the efficient co-crystallization with the matrix substances. In reflectron mode MALDI-TOF-MS sialylated glycopeptides are prone to in-source and metastable

decay.^{192,207} Moreover, poor ionization of sialylated glycopeptides in the positive ion mode further hampers their detection. Interestingly, with DHB and Cl-CCA we observed intense signals for intact sialylated IgG Fc N-glycopeptides in reflectron- and linear-negative ion mode MALDI-TOF-MS. This might be explained by a similarly efficient ionization of neutral and acidic tryptic glycopeptides in the negative ion mode due to acidic groups of the peptide backbones.²⁰⁸ Moreover, the cold nature of the DHB and Cl-CCA matrices²⁰⁰ minimizes analyte fragmentation which is supported by the low intensity of the IgG2 G0F or G1F fragment ion observed at m/z 2396.96 ($[M-H]^-$) or 2398.98 ($[M+H]^+$) (Figure 2 and Figure S-1). Interestingly, the relative abundances of these in-source decay products were even lower with Cl-CCA than with DHB.

Human polyclonal IgG predominantly contains Fc N-glycans with one sialic acid which tends to be more stable during MALDI ionization than multiply sialylated structures. Analysis of intact monosialylated IgG glycopeptides was therefore accomplished by reflectron-negative ion mode MALDI-TOF-MS. In line with literature,^{94,148,192} accurate detection of intact highly sialylated glycopeptides with good intensity was only achieved with linear-negative ion mode MALDI-TOF-MS. The most intense glycopeptide signals for DHB and Cl-CCA were recorded below m/z 5000. Above m/z 5000 the signal strength decreases which is in line with literature data for DHB and CHCA.¹⁹¹

The Cl-CCA preparations showed a good stability when stored at atmospheric pressure but when stored at high vacuum matrix sublimation resulted in relatively fast spot disappearance which is in accordance with the previously reported high vacuum volatility of this matrix.²⁰⁶ While this could be considered a disadvantage, the duration of most automated MALDI-TOF-MS measurements remains far below one minute per sample spot, and thus results will be obtained before any effect of the matrix sublimation is perceived. Furthermore, matrix contamination in the MALDI source is significantly decreased as the sublimation process provides self cleaning of the system by the vacuum pumps.

Macrocrystalline dried droplet preparations of DHB show a distinct distribution of peptides and carbohydrates in large crystals offering the possibility to generate relative clean carbohydrate spectra in presence of contaminating peptides.¹⁹⁷ By contrast, the more homogeneous distribution of compounds in microcrystalline preparations such as obtained with Cl-CCA increase the sensitivity to contaminations. Thus resulting spectra reveal carbohydrate information with additional peptide contamination such as observed by us for the N-glycans released from tryptic fetuin glycopeptides (Figure 5).

Interestingly, when compared to DHB, Cl-CCA resulted in the registration of highly sialylated glycopeptides at relatively high abundance while partially sialylated species showed low signal intensities. The relative intensities of the glycan species observed with Cl-CCA more closely resembled the profiles observed for permethylated N-glycans (Figure S-9) and were more in-line with their previously reported abundance on bovine fetuin¹⁷⁴ than those observed with DHB. This might be explained by less in-source decay of the fully sialylated species with Cl-CCA preparations, though DHB is

reported to be a colder matrix than Cl-CCA.²⁰⁰ Alternatively, the differences in profiles obtained with DHB and Cl-CCA may be caused by different ionization efficiencies of fully sialylated versus partially sialylated and unsialylated structures.

For Cl-CCA high matrix background levels in negative ion mode MALDI-TOF-MS have already been described.²⁰⁰ For positive ion mode measurements, this background has been attributed to high levels of alkali salt contamination in the sample or matrix.^{209,210} The Cl-CCA matrix commercially obtained as a white powder with 95% purity revealed similar levels of alkali salt adducts as the highly purified DHB matrix. Additional purification of the Cl-CCA matrix by recrystallization might further reduce the level of observed sodium and potassium adducts.²⁰⁶ The high level of matrix cluster background with relatively low S/N ratio of released (hydrophilic) N-glycans in negative ion mode MALDI-TOF-MS is probably caused by the low tendency of these analytes to co-crystallize with the relative hydrophobic matrix and this matrix may, therefore, be better suited for labeled glycans. Indeed, Cl-CCA co-crystallized PMP-labeled O-glycans analyzed in positive ion mode revealed superior signals as compared to DHB (see Figure S-10 and S-11). Structural assignment of the detected glycoforms was performed on the basis of literature knowledge.^{204,205} While LC-MS analyses of the PMP-labeled mucin O-glycan samples gave no indications of peeling (data not shown), the unassigned peaks present in the MALDI-TOF-MS spectra indicate that there are still some contaminants or side reaction products present in our purified sample. Nevertheless, we here clearly demonstrated that the use of Cl-CCA has a high potential for the positive ion mode MALDI-TOF-MS analysis of PMP-labeled O-glycans.

In conclusion, we showed that the rationally designed MALDI matrix Cl-CCA allows the sensitive analysis of intact labile sialylated glycopeptides by negative ion mode MALDI-TOF-MS with a low interday variability. The microcrystalline nature of Cl-CCA provided a high shot-to-shot repeatability, resolution and mass accuracy, and offered the possibility to use stainless steel MALDI target plates which are relatively cheap and very robust. This makes Cl-CCA a matrix with high potential in high-throughput MALDI-TOF-MS profiling of glycopeptides. Moreover, we demonstrated the usefulness of Cl-CCA as a matrix for glycan analysis.

Acknowledgements

The authors thank Dr. Oleg I. Klychnikov for making the photographs used in Figure 1. Dr. Thorsten W. Jaskolla and Prof Dr. Michael Karas are acknowledged for kindly providing the Cl-CCA matrix used for the initial pilot experiments. Agnes L. Hipgrave Ederveen and Carolien A.M. Koeleman are acknowledged for preparing and measuring the permethylated fetuin and transferrin N-glycans. The monoclonal antibody used to determine the limit of detection for MALDI-TOF-MS with Cl-CCA or DHB matrix were kindly provided by Hoffmann la Roche. Maurice H.J. Selman thanks Hoffmann la Roche for financial support.

Supporting information

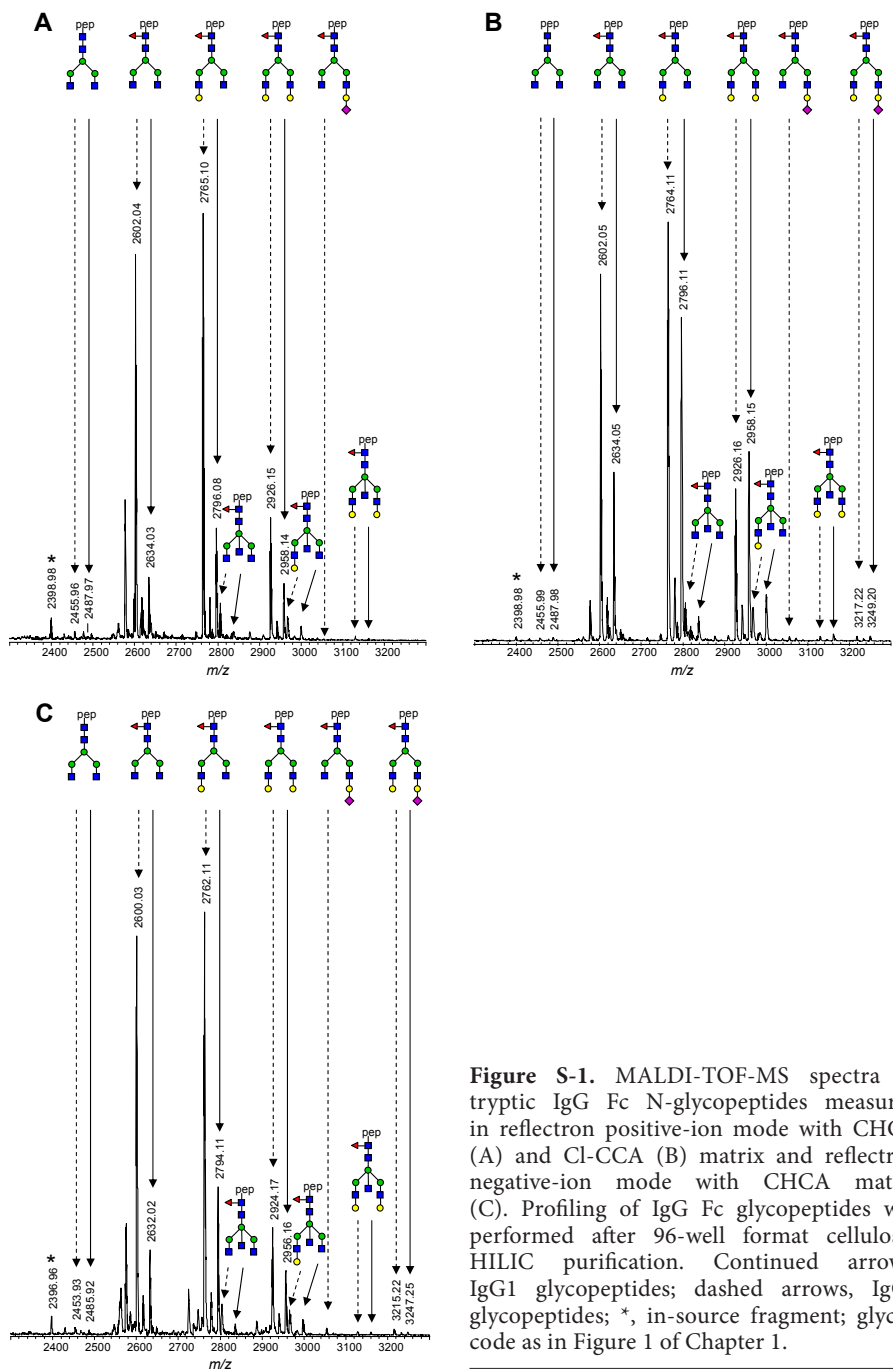


Figure S-1. MALDI-TOF-MS spectra of tryptic IgG Fc N-glycopeptides measured in reflectron positive-ion mode with CHCA (A) and Cl-CCA (B) matrix and reflectron negative-ion mode with CHCA matrix (C). Profiling of IgG Fc glycopeptides was performed after 96-well format cellulose-HILIC purification. Continued arrows, IgG1 glycopeptides; dashed arrows, IgG2 glycopeptides; *, in-source fragment; glycan code as in Figure 1 of Chapter 1.

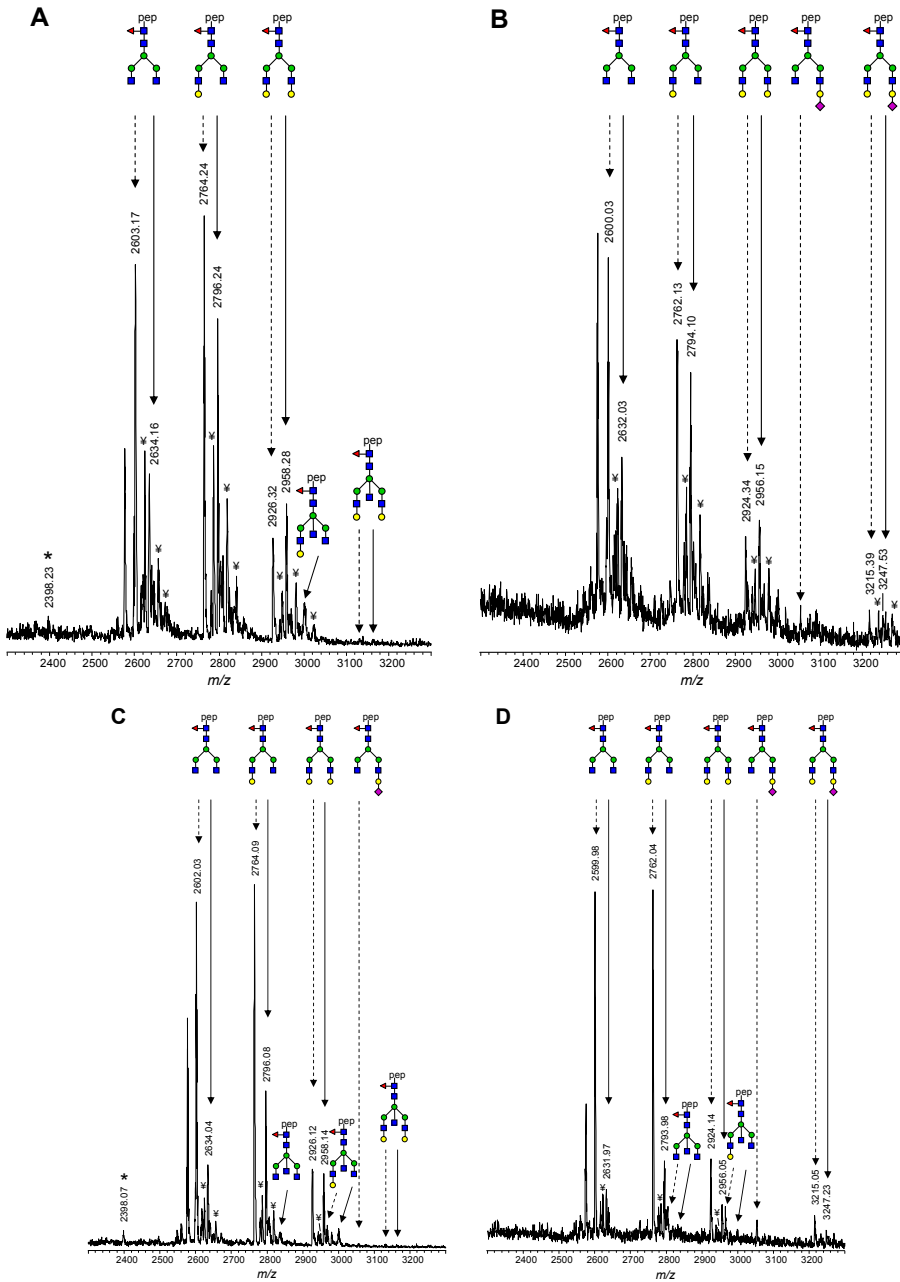


Figure S-2. MALDI-TOF-MS spectra of tryptic IgG Fc N-glycopeptides measured in reflectron-positive ion mode (A, C) and reflectron-negative ion mode (B, D) with THAP (A, B) and THAP/diammonium citrate (C, D) matrix. Profiling of IgG Fc glycopeptides was performed after 96-well format cellulose-HILIC purification. Dashed arrows, IgG2 glycopeptides; solid arrows, IgG1 glycopeptides; *, in-source fragment; ¥, alkali salt adducts; glycan code as in Figure 1 of Chapter 1.

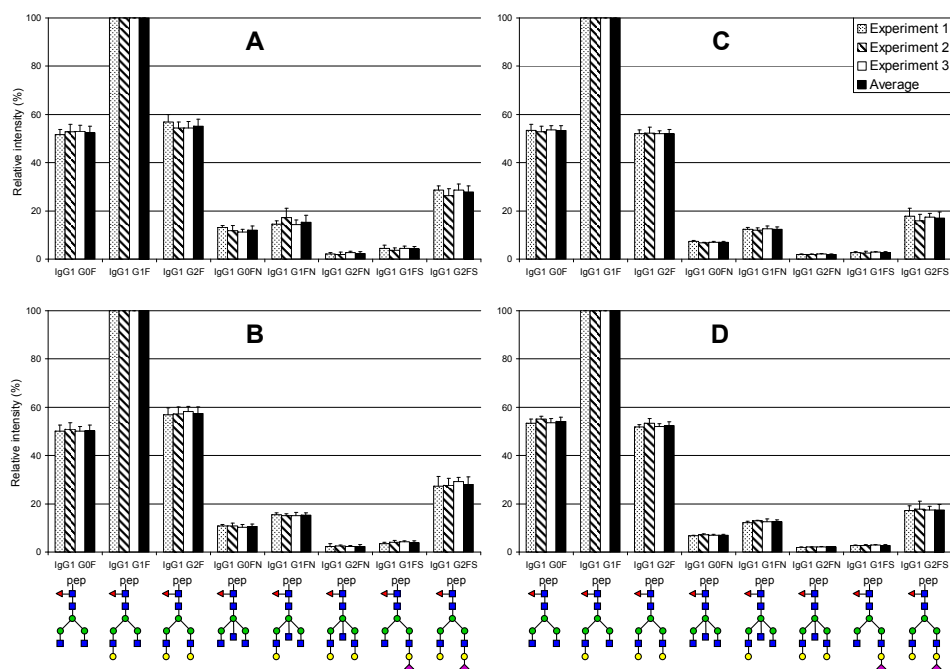


Figure S-3. Repeatability of 96-well cellulose HILIC purification and negative ion mode MALDI-TOF-MS analysis for tryptic IgG1 Fc N-glycopeptides. Analysis of IgG1 glycopeptides with DHB (A, C) or CL-CCA (B, D) was performed by linear- (A, B) and reflectron- (C, D) negative ion mode MALDI-TOF-MS. Relative intensities and standard deviations were determined from three independent experiments comprising eight replicates each. Glycan code as in Figure 1 of Chapter 1.

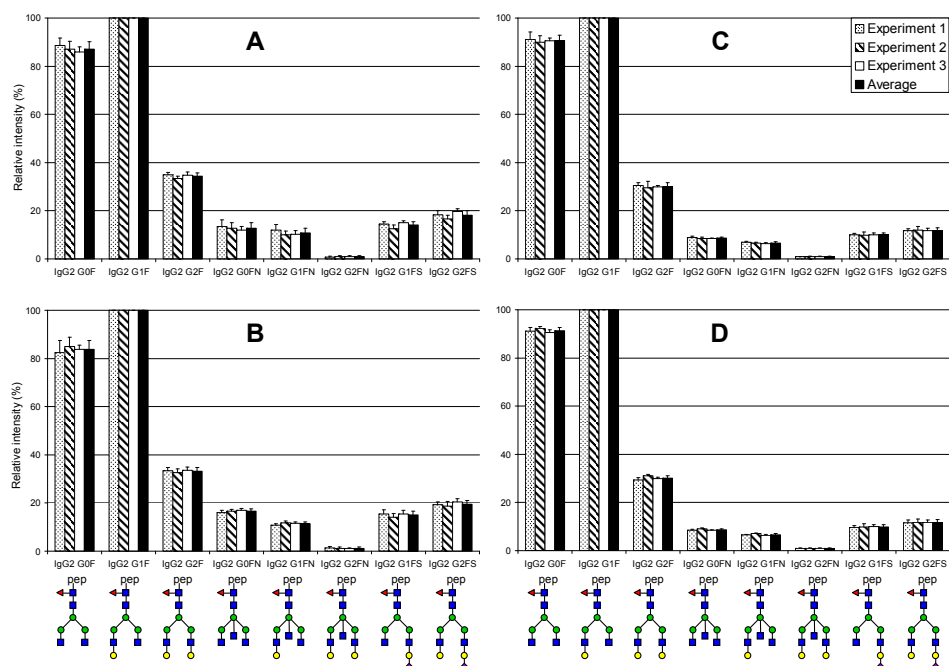


Figure S-4. Repeatability of 96-well cellulose HILIC purification and negative ion mode MALDI-TOF-MS analysis for tryptic IgG2 Fc N-glycopeptides. Analysis of IgG2 glycopeptides with DHB (A, C) or Cl-CCA (B, D) was performed by linear- (A, B) and reflectron- (C, D) negative ion mode MALDI-TOF-MS. Relative intensities and standard deviations were determined from three independent experiments comprising eight replicates each. Glycan code as in Figure 1 of Chapter 1.

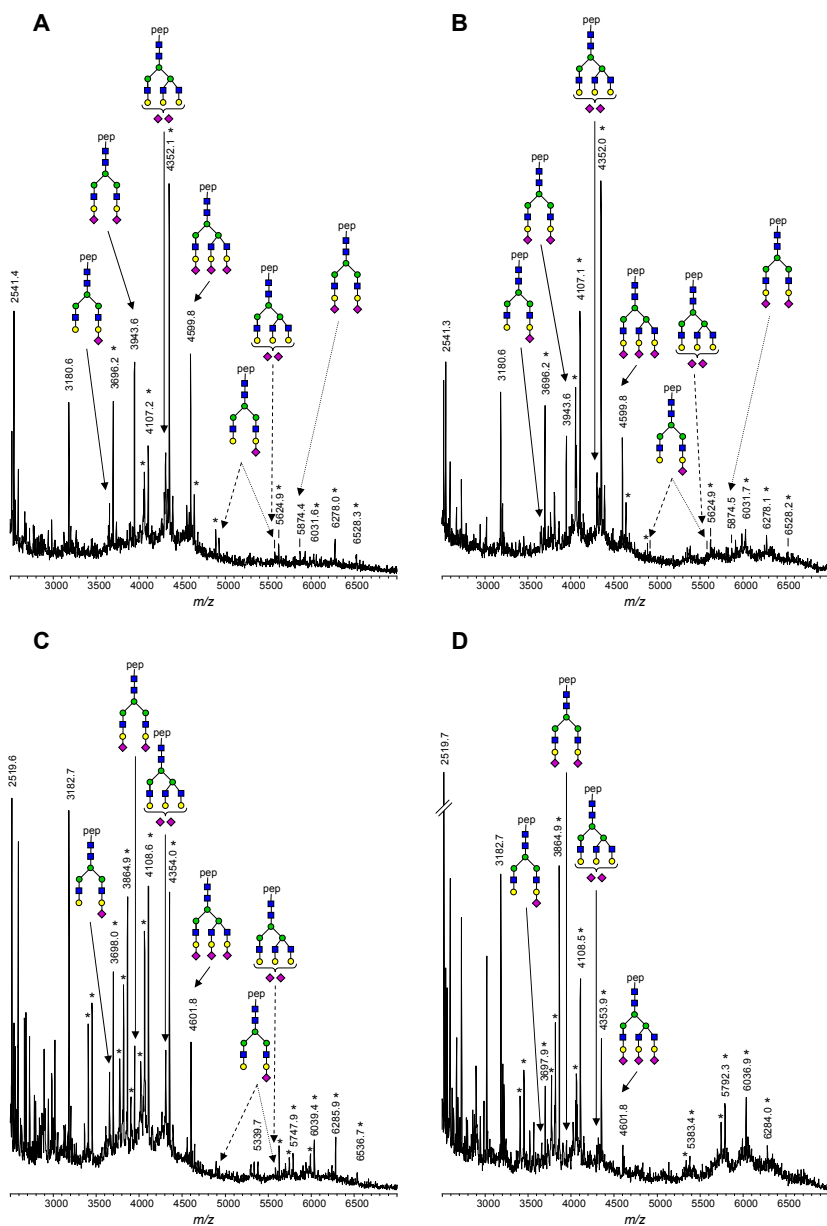


Figure S-5. Reflectron-negative (A, B) and reflectron-positive (C, D) ion mode MALDI-TOF-MS spectra of tryptic bovine fetuin glycopeptides (SwissProt entry number: P12763). Tryptic glycopeptides were purified by cotton HILIC micro-SPE and measured on an AnchorChip target plate with DHB (A, C) and a polished steel target plate with CI-CCA (B, D). Solid arrows, small molecular mass glycopeptide (L_{145} CPDCPLLA P L N DS R_{159}); dashed arrows, intermediate molecular mass glycopeptide (V_{160} VH A VE A L A T F N A ES N GS Y L Q L V E I S R_{187}); dotted arrows, large molecular mass glycopeptide (R_2 PTGE V YD I E I D T LE T T C H V L D P T P L AN C S V R $_{103}$); *, post source decay products; glycan code as in Figure 1 of Chapter 1

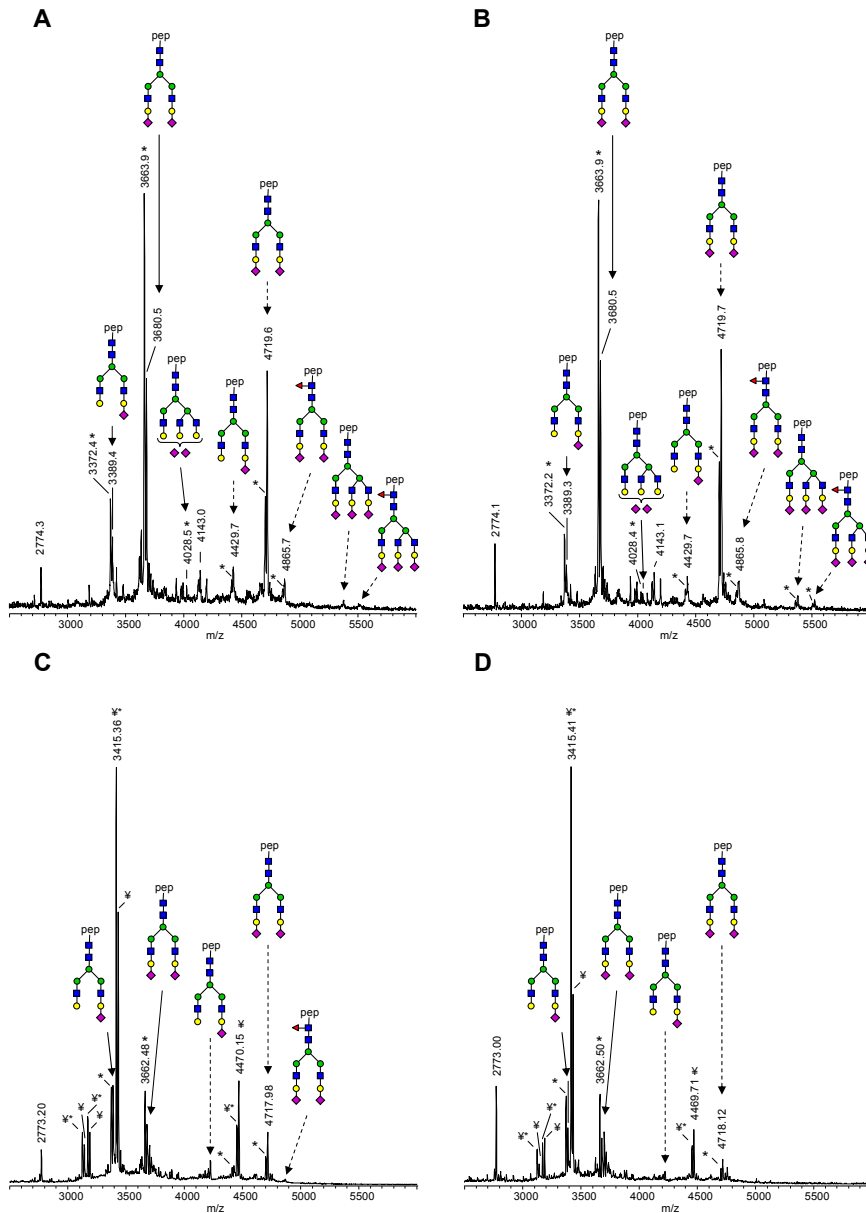


Figure S-6. Linear-negative (A, B) and reflectron-negative (C, D) ion mode MALDI-TOF-MS spectra of tryptic human apo-transferrin glycopeptides (SwissProt entry number: P02787). Tryptic glycopeptides were purified by cotton HILIC micro-SPE and measured on an AnchorChip target plate with DHB (A, C) and a polished steel target plate with Cl-CCA (B, D). Solid arrows, small molecular mass glycopeptide (C_{421} GLVPVLAENYNK $_{433}$); dashed arrows, large molecular mass glycopeptide (Q_{622} QQHLFGSNVTDCSGNFCLFR $_{642}$); ¥, post source decay products; glycan code as in Figure 1 of Chapter 1; *, ammonia loss by N-terminal peptide degradation during proteolytic digestion.¹¹⁰

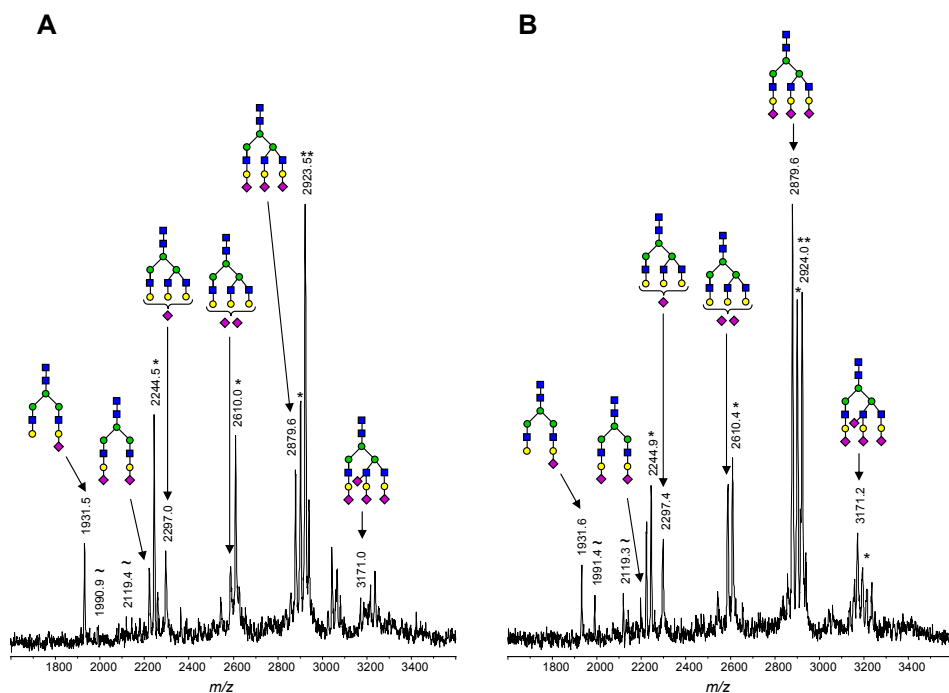


Figure S-7. Linear-negative ion mode MALDI-TOF-MS spectra of PNGase F released bovine fetuin N-glycans. Released N-glycans were purified by cotton HILIC micro-SPE and prepared with THAP (A) and THAP/diammonium citrate (B) on an AnchorChip target plate for analysis. *, Sodium adduct; ~, fetuin peptide $H_{313}TFSGVASVESSSGEAFHVG K_{333}$; glycan code as in Figure 1 of Chapter 1.

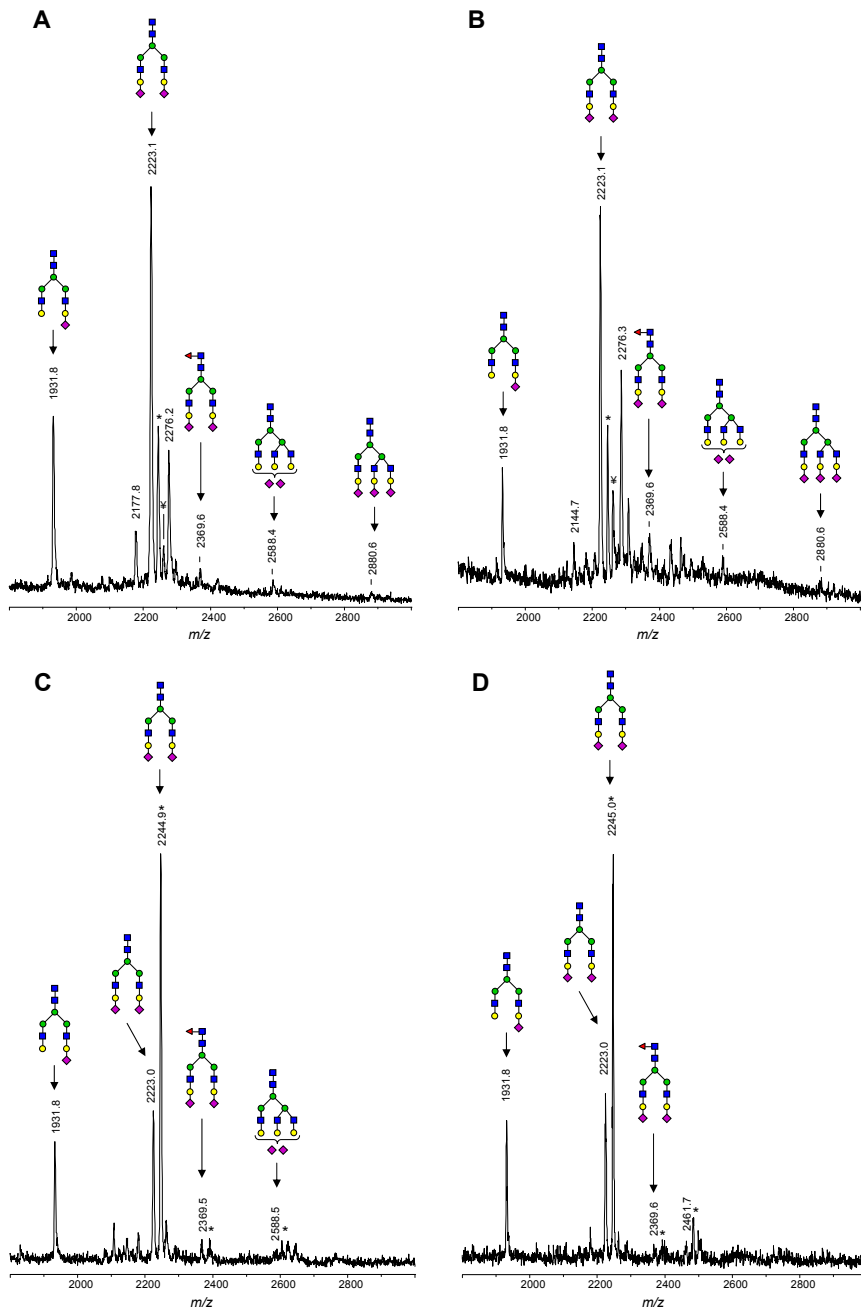


Figure S-8. Linear-negative ion mode MALDI-TOF-MS spectra of PNGase F released human apo-transferrin N-glycans. Released N-glycans were purified by cotton HILIC micro-SPE and prepared on an AnchorChip with DHB (A), THAP (C) or THAP/diammonium citrate (D) or on a polished steel target plate with Cl-CCA (B) for analysis. *, Sodium adduct; ‡, potassium adduct; glycan code as in Figure 1 of Chapter 1.

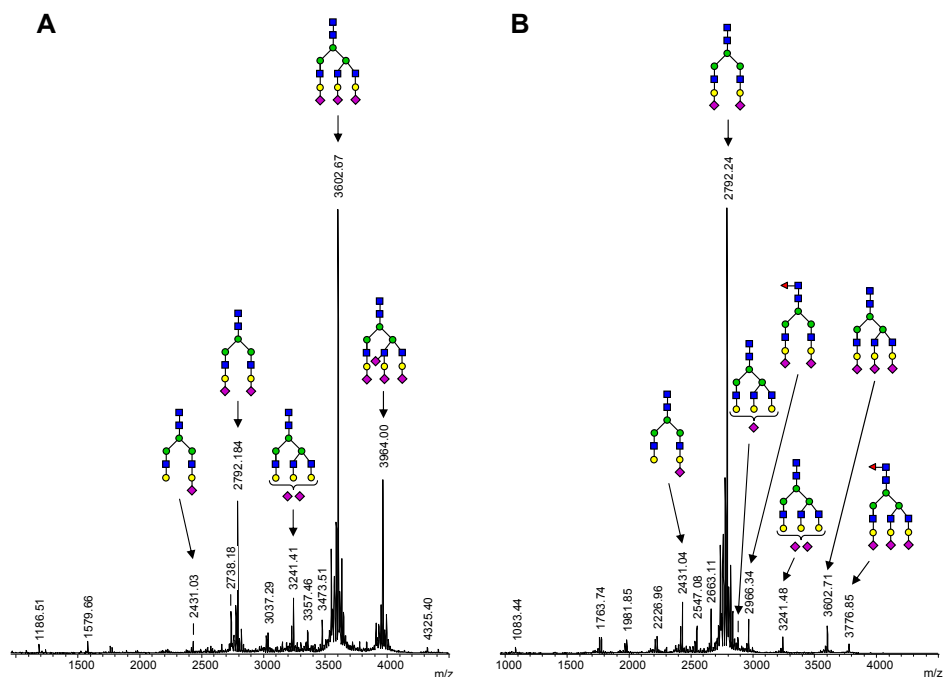


Figure S-9. Reflectron-positive ion mode MALDI-TOF-MS spectra of released and permethylated fetuin (A) and transferrin (B) N-glycans prepared on an AnchorChip with DHB. Glycan code as in Figure 1 of Chapter 1.

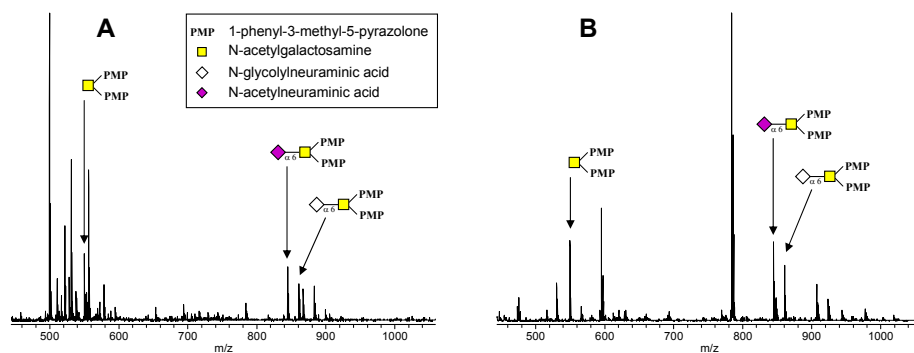


Figure S-10. Reflectron-positive ion mode MALDI-TOF-MS spectra of released mucin O-glycans. Released and PMP-labeled O-glycans were purified with a reversed phase ziptip and prepared on a AnchorChip plate with DHB (A) or a polished steel plate with Cl-CCA (B) for analysis.

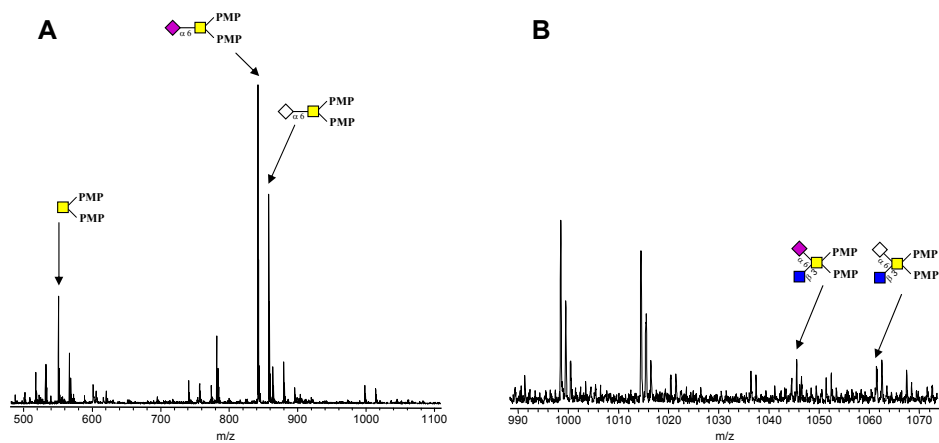


Figure S-11. Reflectron-positive ion mode MALDI-TOF-MS spectra of released mucin O-glycans prepared on a AnchorChip plate with Cl-CCA. Released and PMP-labeled O-glycans were purified with a reversed phase ziptip and spotted directly for analysis. A. high abundant O-glycans, B. zoom-in of low abundant O-glycans. Glycan code as in Figure S-10.

Changes in antigen-specific IgG1 Fc N-glycosylation upon influenza and tetanus vaccination

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Mol. Cell. Proteomics, 2011, M111.014563, In-press.

Abstract

Antibody effector functions have been shown to be influenced by the structure of the Fc N-glycans. Here we studied the changes in plasma or serum IgG Fc N-glycosylation upon vaccination of 10 Caucasian adults and 10 African children. Serum/plasma IgG was purified by affinity chromatography prior to and at two time points after vaccination. Fc N-glycosylation profiles of individual IgG subclasses were determined for both total IgG and affinity-purified anti-vaccine IgG using a recently developed fast nanoLC-ESI-MS method. While vaccination had no effect on the glycosylation of total IgG, anti-vaccine IgG showed increased levels of galactosylation and sialylation upon active immunization. Interestingly, the number of sialic acids per galactose moiety (SA/Gal) increased during the vaccination time course, suggesting a distinct regulation of galactosylation and sialylation. In addition we observed a decrease in the level of IgG1 bisecting N-acetylglucosamine while no significant changes were observed for the level of fucosylation. Our data indicate that depending on the vaccination time point the infectious agent will encounter IgGs with different glycosylation profiles which are expected to influence the antibody effector functions relevant in immunity.

Introduction

Millions of individuals are vaccinated worldwide each year to stimulate the adaptive immune system to produce protective antibodies as well as T-cell responses against a variety of pathogens. Vaccination with attenuated microbe strains and purified proteins results in lymphocyte sensitization, cytokine release, and the production of immunoglobulins (Igs) which may provide long-term immunity.

The most abundant Ig class in the humoral immune response is IgG being present at concentrations of approximately 10 mg/ml in plasma and serum.^{1,2} IgGs are glycoproteins, and their glycosylation is known to modulate antibody activity and effector mechanisms.^{19,35,43,44,146} Four different IgG subclasses are present in humans (IgG1-4). IgGs consist of two heavy and two light chains. The two light chains together with the N-terminal domains (V_H and C_{H1}) of the two heavy chains form the fragment antigen binding (Fab) moiety, whilst the fragment crystallisable (Fc) moiety is formed by the C-terminal domains (C_{H2} and C_{H3}) of the two heavy chains. A single biantennary, often core fucosylated N-glycan is attached to the asparagine residue at position 297 in the C_{H2} domain of the heavy chains. These N-glycans vary in the number of antenna galactoses and may carry a sialic acid on one of the antennae. Part of the N-glycans contain a bisecting N-acetylglucosamine (bisecting GlcNAc).¹⁴⁷

Recently, some B-cell stimuli have been identified which resulted in changes in antibody glycosylation and indicated a pronounced short-term regulation of IgG glycosylation in humans.⁵⁷ *In-vitro* stimulation of B-cells with the environmental factor all-trans retinoic acid resulted in the expression of IgG1 with decreased galactosylation within a time-range of several days, while increased galactosylation and reduced bisecting GlcNAc have been observed after stimulation with CpG oligodeoxynucleotide (stimulates the innate immune system) or interleukin 21 (stimulates the adaptive immune system).⁵⁷

Hitherto, induction of specific glycosylation patterns of IgGs upon immunization has only been shown in animal experiments.^{34,35,211} Specific pathogen free CBA/Ca mice immunized with bovine serum albumin (BSA) in incomplete Freund's adjuvant showed an initial decrease in galactose content on anti-BSA IgG with increasing antibody titer which returned to original levels when titers fell.³⁴ In a murine nephrotoxic serum nephritis model, total IgG sialylation has been shown to reduce drastically in mice pre-sensitized with sheep IgG and challenged with sheep anti-mouse glomerular basement membrane preparation compared to unimmunized controls.³⁵ This suggests that the sialylation change may provide a switch from innate anti-inflammatory activity in the steady state to an adaptive pro-inflammatory response. Repeated immunization of male ICR mice with ovalbumin (OVA) in physiological saline resulted in an increase of the fucose content on anti-OVA IgG, while mannosylation, galactosylation and sialylation were unaffected.²¹¹ These animal studies demonstrate that upon immunological challenge glycosylation of the produced antibodies differ.

Measurement of IgG glycosylation at the glycopeptide level ensures specificity as it allows the assignment of glycan structures to the Fc portions of individual IgG subclasses, which is important since Fc glycosylation and Fab glycosylation appear to have very distinct functions.²¹² Due to the high sensitivity of the mass spectrometric detection it is possible to set up affinity-based microtitration well plate IgG capturing and purification assays as modifications of (commercially available) enzyme-linked immunosorbent assays and combine them successfully with IgG glycosylation profiling of glycopeptides.⁵⁶ This allows glycan profiling at the level of antigen-specific IgG. Interestingly, antigen-specific IgGs have repeatedly been shown to be glycosylated in a very distinct manner that differs considerably from the pool of total IgG.^{22,24,45,56}

Here, we describe IgG glycosylation changes induced by vaccination in humans. We analyzed the Fc glycosylation of IgG1 induced by vaccination against Mexican flu (Caucasian adults), seasonal flu (African children) and tetanus (African children). Consistently we observe a transient increase of both galactosylation and sialylation, together with a decrease of the incidence of bisecting GlcNAc. This glycosylation time course is specifically observed for the vaccine-induced IgG1 whilst the glycosylation of total IgG1 is unaffected. On the basis of the known association of IgG glycosylation features with antibody efficacy in *in-vitro* assays and animal models,^{35,43,44,47,48,213,214} we expect that the specific IgG1 glycosylation features observed upon vaccination will influence antibody effector functions.

Experimental section

Study cohort. The study cohort is described in Table 1. From 10 healthy Caucasians who were vaccinated twice (at day 0 and at day 21) with MF59-adjuvanted 2009 pandemic influenza A(H1N1) (Focetria 2009, Novartis Vaccines and Diagnostics, Rosia, Italy), serum was obtained at day 0 (just before vaccination), day 21 (just before the second dose) and day 56.²¹⁵ This study was approved by the ethics committee of Leiden University Medical Center (protocol number 09.187). Subjects provided written informed consent for participation in the study and for the use of stored serum samples for the purpose of this study.²¹⁵

From 10 Gabonese children heparin plasma was obtained prior to vaccination (day 0) and at day 14 and 28 after vaccination with Begrivac 2004/2005 (Chiron Behring GmbH, Marburg, Germany).²¹⁶ Simultaneously with the influenza vaccination, the African children were vaccinated with a tetanus toxoid booster (NIPHE, Bilthoven, The Netherlands). For six of the ten children enough material was available to additionally evaluate tetanus specific IgG Fc N-glycosylation changes. Three girls and all boys were tested positive for helminth infection and one girl was tested positive for malaria. The study was approved by the ethics committee of the International Foundation of the Albert Schweitzer Hospital in Lambaréné. Written informed consent was obtained from the parents or legal guardians of each child prior to participation in the study.²¹⁶

Table 1. Human sample cohort.

Group	Sex	Counts	Vaccination	Age (years)			
				Youngest	Oldest	Mean	STD
Caucasian	Female	7	Influenza (Focetria)	33	58	45.7	9.9
	Male	3	Influenza (Focetria)	26	59	40.7	16.8
African	Female	6	Influenza (Begrivac)	7	11	8.8	1.6
	Male	4	Influenza (Begrivac)	8	10	9.0	0.8
African	Female	4	Tetanus	7	11	9.3	1.71
	Male	2	Tetanus	9	10	9.5	0.71

Preparation of antigen-specific affinity beads. For the preparation of antigen-specific beads pooled vaccine doses of Focetria (20 doses), Begrivac 2004/2005 (4 doses) and tetanus toxoid (10 doses) were buffered 4:1 (v:v) with 0.2 M sodium bicarbonate (Fluka, Steinheim, Germany) containing 0.5 M sodium chloride (Merck, Darmstadt, Germany). NHS-activated Sepharose 4 Fast Flow beads (GE Healthcare, Uppsala, Sweden) were washed 3 times with 10 volumes of ice-cold 1 mM hydrochloric acid (HCl; Merck), and to 200-400 μ l of the beads buffered Focetria, Begrivac or tetanus toxoid was applied. The antigens were immobilized overnight at 4°C under continuous shaking followed by a 4 h blocking of residual NHS groups at RT with 0.1 M tris(hydroxymethyl) aminomethane (Tris; Roche Diagnostics, Mannheim, Germany) brought to pH 8.5 with HCl (Merck). Beads were washed 3 times with alternating pH using 0.1 M glacial acetic acid (Merck) with 0.5 M sodium chloride (pH 4-5) and Tris-HCl (pH 8.5) and stored in 0.1 M Tris-HCl (pH 8.5) with 20% ethanol at 4°C until usage.

IgG glycosylation analysis. Human polyclonal IgGs (IgG1, 2 and 4) were captured from 2 μ l plasma or serum by affinity chromatography with Protein A-Sepharose™ Fast Flow beads (GE Healthcare) in 96-well plates as described previously.¹⁴⁸ Vaccine-specific IgGs were purified from 20 μ l human plasma or serum by incubation with 3-5 μ l of the immobilized tetanus toxoid or influenza antigen beads in 96-well filter plates for one hour. Captured antibodies were washed with 3 x 200 μ l PBS, 3 x 200 μ l water, eluted with 100 μ l of 100 mM formic acid (Fluka) and dried by vacuum centrifugation. Purified IgGs (total and vaccine-directed) were dissolved in 20 μ l of 50 mM ammonium hydrogencarbonate buffer (Fluka), and shaken for 10 minutes. To minimize potential autolysis and missed cleavages, lyophilized sequencing grade modified trypsin (Promega, Madison, WI) was dissolved in ice-cold 1 mM acetic acid (Merck, Darmstadt, Germany), and immediately 20 μ l was added to the IgG samples. Samples were shaken for 1 minute, checked for the correct pH (>6) and incubated overnight at 37°C. The digests were centrifuged at 4000 rpm for 5 min and aliquots (250 nl for total IgG, 5000 nl for vaccine specific IgG) were analyzed by fast nanoLC-ESI-MS²¹⁷ on a Ultimate 3000 HPLC system (Dionex Corporation, Sunnyvale, CA) equipped with a Dionex Acclaim PepMap100 C18 (5 μ m

particle size, 5 mm x 300 µm i.d.) trap column and an Ascentis Express C18 nano column (2.7 µm HALO fused core particles, 50 mm x 75 µm i.d.; Supelco, Bellefonte, USA), which were coupled to a micrOTOF-Q mass spectrometer (Bruker Daltonics, Bremen, Germany) by a sheath-flow-ESI sprayer (capillary electrophoresis ESI-MS sprayer; Agilent Technologies, Santa Clara, USA).²¹⁷ Scan spectra were recorded from 300 to 2000 dalton with 2 average scans at a frequency of 1 Hz. The Ultimate 3000 HPLC system and the Bruker micrOTOF-Q were respectively operated by Chromeleon Client version 6.8 and micrOTOF control version 2.3 software. Liquid chromatography-mass spectrometry datasets were internally calibrated using a list of known glycopeptides and exported to the open mzXML using Bruker DataAnalysis 4.0 in batch mode. Data processing was performed with the in-house developed software msalign2,¹⁵³ a simple warping script in AWK,¹⁵⁴ “Xtractor2D” and Microsoft Excel. The software and ancillary scripts are freely available at www.ms-utils.org/Xtractor2D. Relative intensities of 46 glycopeptide species (Table 2) derived from IgG1 (18 glycoforms), IgG4 (10 glycoforms), and IgG2 (18 glycoforms) were obtained by integrating and summing three isotopic peaks followed by normalization to the total subclass-specific glycopeptide intensities.

On the basis of the normalized intensities for the various IgG Fc N-glycoforms the level of galactosylation, sialylation, bisecting N-acetylglucosamine and fucosylation was calculated. The level of galactosylation was calculated according to the formula $(G1F + G1FN + G1FS + G1FNS + G1 + G1N + G1S) * 0.5 + G2F + G2FN + G2FS + G2FNS + G2 + G2N + G2S$ for the IgG1 and IgG2 subclasses and $(G1F + G1FN + G1FS + G1FNS) * 0.5 + G2F + G2FN + G2FS + G2FNS$ for the IgG4 subclass. The prevalence of IgG sialylation was determined by summation of all sialylated Fc N-glycopeptide species (G1FS, G2FS, G1FNS, G2FNS, G1S and G2S for IgG1 and IgG2, and G1FS, G2FS, G1FNS and G2FNS for IgG4). The number of sialic acid moieties present on the galactose moieties (SA/Gal) is calculated by dividing the prevalence of IgG sialylation by $2 * \text{the level of galactosylation}$. The incidence of bisecting N-acetylglucosamine is obtained by summing all bisected Fc N-glycopeptide species (G0FN, G1FN, G2FN, G1FNS, G2FNS, G0N, G1N and G2N for the IgG1 and IgG2 subclass or G0FN, G1FN, G2FN, G1FNS and G2FNS for the IgG4 subclass). The percentage of IgG1 and IgG2 fucosylation was determined by summing the relative intensities of all fucosylated Fc N-glycopeptide species (G0F, G1F, G2F, G0FN, G1FN, G2FN, G1FS and G2FS). For the IgG4 subclass no fucosylation level was determined as the afucosylated species remained below the limit of detection.

Statistical analysis. Differences between the 3 vaccination time points were evaluated using the Friedman test. Uncorrected *p*-values < 0.05 were considered statistically significant. In depth analyses of the differences between protein A (total IgG1) and vaccine-specific purified IgG1 were performed with the Wilcoxon Signed Rank test. *p*-Values were adjusted for multiple comparisons by Bonferroni correction, and *p*-values < 0.013 were considered statistically significant. Data evaluation and statistical analyses were performed with Microsoft Excel and SPSS 16.0, respectively.

Table 2. Glycoforms of human plasma IgG detected by nanoLC-ESI-MS.

Glycan species	IgG1 P01857 ^b		IgG2 P01859 ^b		IgG4 P01861 ^b	
	E ₂₉₃ EQYNSTYR ₃₀₁ ^c		E ₂₉₃ EQFNSTFR ₃₀₁ ^c		E ₂₉₃ EQFNSTYR ₃₀₁ ^c	
	[M+2H] ²⁺	[M+3H] ³⁺	[M+2H] ²⁺	[M+3H] ³⁺	[M+2H] ²⁺	[M+3H] ³⁺
No glycan	595.260	397.176	579.265	386.513	587.262	391.844
G0F ^c	1317.527	878.687	1301.532	868.024	1309.529	873.356 ^{a1}
G1F	1398.553	932.705	1382.558	922.042	1390.556	927.373 ^{a2}
G2F	1479.580	986.722	1463.585	976.059	1471.582	981.391
G0FN	1419.067	946.380	1403.072	935.717	1411.069	941.049 ^{a3}
G1FN	1500.093	1000.398	1484.098	989.735	1492.096	995.066 ^{a4}
G2FN	1581.119	1054.416	1565.124	1043.752	1573.122	1049.084
G1FS	1544.101	1029.737	1528.106	1019.073	1536.104	1024.405 ^{a5}
G2FS	1625.127	1083.754	1609.132	1073.091	1617.130	1078.423
G1FNS	1645.641	1097.430	1629.646	1086.766	1637.643	1092.098
G2FNS	1726.667	1151.447	1710.672	1140.784	1718.670	1146.116
G0	1244.498	830.001	1228.503	819.338	-	-
G1	1325.524	884.019	1309.529	873.356 ^{a1}	-	-
G2	1406.551	938.036	1390.556	927.373 ^{a2}	-	-
G0N	1346.038	897.694	1330.043	887.031	-	-
G1N	1427.064	951.712	1411.069	941.049 ^{a3}	-	-
G2N	1508.090	1005.730	1492.096	995.066 ^{a4}	-	-
G1S	1471.072	981.051	1455.077	970.387	-	-
G2S	1552.098	1035.068	1536.104	1024.405 ^{a5}	-	-

^{a1} – ^{a5}isomeric glycopeptide species of IgG4 and IgG2.

^bSwissProt entry number

^ctryptic IgG glycopeptide sequence

^dglycan structural features are given in terms of number of galactoses (G0, G1, G2), fucose (F), bisecting N-acetylglucosamine (N), and N-acetylneuraminic acid (S).

Results

To evaluate IgG Fc N-glycosylation changes upon vaccination tryptic IgG glycopeptides prepared from human plasma or serum were analyzed using a recently established fast nanoLC-ESI-MS method.²¹⁷ An optimized tryptic cleavage procedure was applied, and no missed tryptic cleavages were observed. On the basis of literature knowledge of IgG N-glycosylation^{3,4,9,10,79} the nanoLC-ESI-MS method allowed unambiguous assignment of 46 IgG glycopeptides species (18 glycoforms of IgG1, 10 of IgG4, and 18 of IgG2; Table 2). Glycoforms having 5 N-acetylhexosamine residues were interpreted as bisected species.^{3,4,9,10,79} In agreement with literature data, N-acetylneuraminic acid was the only form of sialic acids observed in our analysis.^{3,4,9,10,79} The fast nanoHPLC separation together with the integration and summation of multiple isotopic peaks for

each assigned IgG Fc N-glycopeptide species provides subclass-specific glycosylation information with good precision.²¹⁷ No indications for the presence of trypsin missed cleavages were obtained as a result of the optimized digestion protocol and were therefore not included in the data.

Fc N-glycosylation profiles of total IgG and antigen affinity captured IgG were evaluated for 10 Caucasian adults and 10 African children prior to vaccination and at several time points after vaccination. For each individual similar total IgG1 Fc N-glycopeptide profiles were observed at all vaccination time points (typical example Figure 1A-C; Figure S-1A-C). By contrast, antigen affinity-purified IgG1 showed changes in Fc N-glycopeptide profiles with time (typical example Figure 1D-F; Figure S-1D-I). Higher intensities for galactosylated glycoforms were observed for Caucasian adults at day 21 and 56 of influenza vaccination as compared to day 0 (Figure 1E-F). Similarly, in African children galactosylated glycoforms for influenza and tetanus affinity-purified IgG1 were higher at day 14 and 28 than before vaccination (Figure S-1). Only very low signals were obtained for IgG4 and IgG2 Fc N-glycopeptides in vaccine affinity purified samples, and no changes of these profiles were observed with

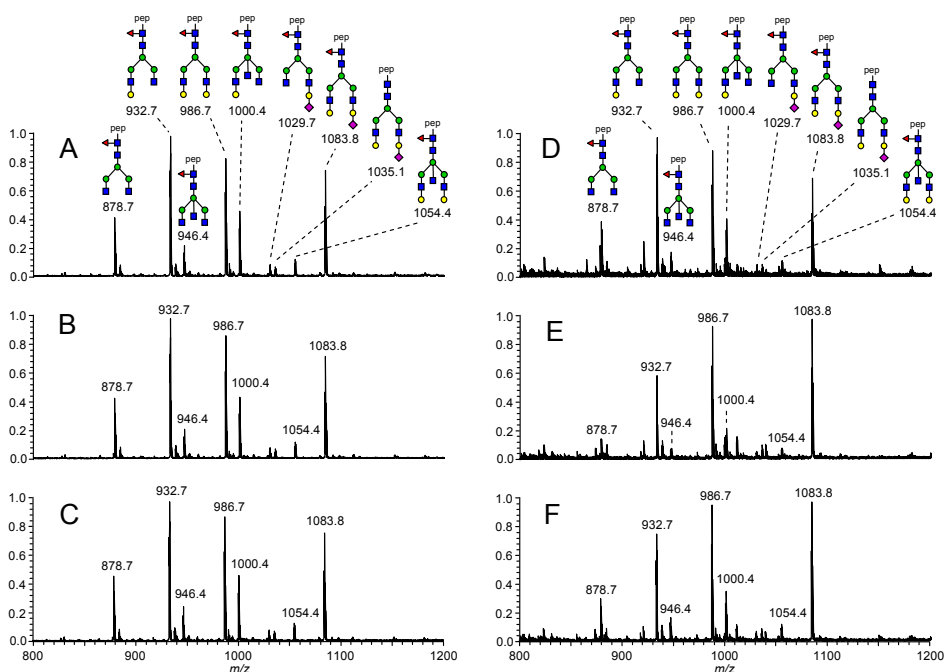


Figure 1. Nano-RPLC-ESI-MS profiles (sum spectra of 1 min) of tryptic IgG1 glycopeptides purified from Caucasian plasma by protein A (A-C) and influenza vaccine (D-F) affinity chromatography at day 0 (A and D), day 21 (B and E), and day 56 (C and F) after vaccination. Glycan code as in Figure 1 of Chapter 1.

time. We, therefore, excluded IgG4 and IgG2 glycosylation from further analysis. In the following, the vaccination-associated changes in IgG1 fucosylation, galactosylation, sialylation, and the incidence of bisecting GlcNAc are described.

Fucosylation. Differences between total and antigen affinity-purified IgG1 were evaluated using the Wilcoxon Signed Rank test. Only the day 0 time point of Begrivac affinity-purified IgG1 of African children showed a significantly lower fucosylation compared with total IgG1 (p -value = 0.007; Table S-1, medians available in Table 3 and 4). Next we looked for longitudinal changes in IgG1 Fc fucosylation by evaluating the three time points with the Friedman test. Total and antigen affinity-purified IgG1 of Caucasian adults (Figure 2A, F) and African children (Figure 3A, F, K) did not show any changes in Fc fucosylation during the influenza or tetanus vaccination time courses.

Galactosylation. In Caucasians and Africans the median level of galactosylation for total IgG1 at day 0 (medians available in Table 3 and 4) was significantly lower than for the influenza affinity purified IgG1 (p -values = 0.005; Table S-1). At the two time points after vaccination (day 21 and 56 for Caucasians, day 14 and 28 for Africans) the level of galactosylation for total IgG1 remained significantly lower compared with the corresponding time points of influenza specific purified IgG1 (p -values = 0.005; Table S-1). For the difference in galactosylation between total IgG1 and tetanus affinity-purified IgG1 p -values of 0.028 were observed which were considered to be non-significant after correction for multiple testing. The Friedman test showed changes in galactosylation upon vaccination of Caucasian adults (Figure 2B, G) and African children (Figure 3B, G, L): antigen affinity-purified IgG1 showed a significant increase in the level of Fc galactosylation (p -value < 0.01; Tables 3 and 4) while no change was observed for total IgG. More specifically the level of galactosylation of influenza affinity purified IgG1 of Caucasians was significantly increased from 62.1% (median) at day 0 to 72.9% (median) at day 21 (p -value = 0.005). At day 56 the level of galactosylation (median = 68.9%) remained significantly elevated compared with day 0 (p -value = 0.005) and showed a tendency towards lower IgG1 galactosylation levels than at day 21 (p -value = 0.047). For African children the median levels of galactosylation of influenza affinity purified IgG1 significantly increased from 56.0% at day 0 to 74.3% at day 14 (p -value = 0.005) and 67.9% at day 28 (p -value = 0.007). At day 28 the level of IgG1 galactosylation was significantly lower compared with day 14 (p -value = 0.005). For tetanus specific purified IgG1 the Friedman test showed a significant change in time for the level of galactosylation (p -value = 0.006; Table 4). Evaluation of the specific differences between the time points with the Wilcoxon Signed Rank test showed a trend towards higher levels of galactosylation upon vaccination of the 6 children with the tetanus booster (median at day 0 = 51.3%, day 14 = 64.7% and day 28 = 62.1%; p -value = 0.028). In accordance, at the level of the individual glycoforms influenza and tetanus vaccination resulted in a significant decrease of the G0F species, while the G2F and G2FS species were significantly increased (p -values < 0.01; Table S-2 and 3).

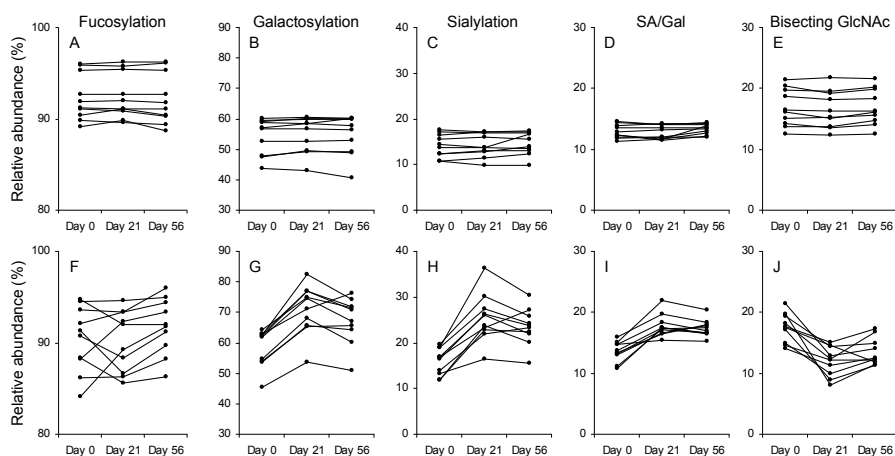


Figure 2. Change in IgG1 glycosylation upon influenza vaccination of Caucasian adults. For total IgG1 (A-E) and antigen (F-J) affinity-purified IgG1 the levels of fucosylation (A, F), galactosylation (B, G), sialylation (C, H), SA/Gal (D, I) and bisecting N-acetylglucosamine (E, J) are given.

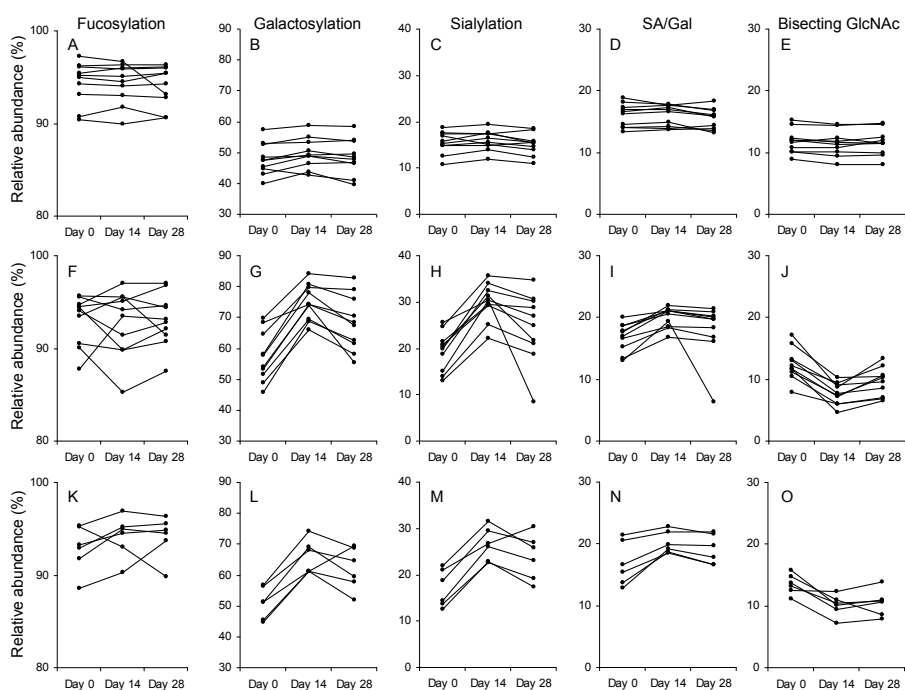


Figure 3. Change in IgG1 glycosylation upon influenza and tetanus vaccination of African children. For total IgG1 (A-E), and influenza (F-J) and tetanus (K-O) affinity-purified IgG1 the levels of fucosylation (A, F, K), galactosylation (B, G, L), sialylation (C, H, M), SA/Gal (D, I, N) and bisecting N-acetylglucosamine (E, J, O) are given.

Sialylation. At day 0 total IgG1 of African children showed a lower median level of sialylation (medians available in Table 4) than influenza affinity-purified IgG1 (p -value < 0.01 ; Table S-1). For both Africans and Caucasians the median levels of sialylation for influenza affinity-purified IgG1 at time points 2 and 3 were significantly higher compared with total IgG1 (p -value < 0.01 ; Table S-1). Influenza affinity-purified IgG1 showed a change in the level of sialylation during vaccination for Caucasian (Figure 3C, H) and African (Figure 3C, H, M) individuals (Friedman test p -value < 0.001 ; Table 3 and 4). The Wilcoxon Signed Rank test revealed a significant increase in the median level of sialylation of influenza affinity purified IgG1 from 16.7% at day 0 to 24.9% at day 21 (p -value = 0.005) in Caucasian adults, which remained elevated with 23.6% at day 56 (p -value = 0.005). No significant difference was observed in the level of sialylation between day 28 and 56. For the African children a significant increase in the median level of sialylation of influenza affinity purified IgG1 was observed between day 0 (median = 20.1%) and day 14 (median = 30.4%; p -value = 0.005), while day 28 (median = 25.9%) only showed a tendency towards higher levels (p -value = 0.022). The level of IgG1 sialylation at day 28 was significantly decreased compared with day 14 (p -value = 0.005). For the 6 African children with additional tetanus boost vaccination the Friedman test showed a tendency towards changed levels of sialylation of the tetanus affinity purified IgG1 (p -value = 0.06; Table 4).

Next we evaluated the number of sialic acids per galactose moiety (SA/Gal; medians available in Table 3 and 4). At day 0 the number of SA/Gal did not significantly differ between total IgG1 and antigen affinity purified IgG1 (influenza and tetanus) (Table S-1). By contrast, at the time points after vaccination the number of SA/Gal was significantly higher for influenza affinity-purified IgG1 than for total IgG1 (p -values < 0.01 ; Table S-1). No significant difference in the number of SA/Gal was reached for the corresponding time points of total IgG1 and tetanus affinity-purified IgG1. Changes in the number of SA/Gal during the vaccination time course were evaluated with the Friedman test. Upon vaccination more sialic acid moieties were found per galactose independent of the ethnicity (Figure 2D, I, Figure 3D, I, N, Table 3 and Table 4). In depth analysis of the data for Caucasians adults with the Wilcoxon Signed Rank test showed increased ratios at days 28 and 56 as compared with day 0 (p -value = 0.005). For influenza affinity purified IgG1 of African children this increase was significant between day 0 and day 14 (p -value = 0.005), but not between day 0 and day 28. Likewise, for the six African children additionally vaccinated against tetanus Friedman's test indicated that there was a significant change in the amount of SA/Gal (Table 4). However, significance was not reached for the comparison of the different time points with the Wilcoxon Signed Rank test.

Incidence of bisecting N-acetylglucosamine. Finally we evaluated the level of bisecting N-acetylglucosamine and compared the obtained medians of total IgG1 for each time point (medians available in Tables 3 and 4) with the corresponding time

points after vaccine affinity purification. At day 0 no significant differences were observed between total IgG1 and antigen affinity-purified IgG1 (Table S-1). After vaccination, influenza affinity-purified IgG1 showed a significantly lower level of bisecting N-acetylglucosamine compared with total IgG1 at the corresponding time points (p -value < 0.01; Table S-1). By contrast, significance was not reached when we compared the corresponding time points between total IgG1 and tetanus affinity-purified IgG1. After influenza vaccination all individuals showed a decrease in the level of bisecting N-acetylglucosamine on influenza affinity-purified IgG1 (Figure 2E, J and Figure 3E, J, O) (Friedman test p -value < 0.001; Table 3 and 4). This decrease did not reach statistical significance for tetanus affinity-purified IgG1. The Wilcoxon Signed Rank test revealed that in Caucasian adults the median level of bisecting N-acetylglucosamine on influenza affinity purified IgG1 decreased from 17.6% at day 0 to 12.2% at day 21 (p -value = 0.005). The median level at day 56 was comparable (12.5%) to the level at day 21. For the African children the level of bisecting N-acetylglucosamine on influenza affinity purified IgG1 decreased from a median of 11.9% at day 0 to 7.5% at day 14 (p -value = 0.005). The level of bisecting N-acetylglucosamine at day 28 were significantly lower than at day 28 (median = 10.0%) than at day 0 (p -value = 0.005) yet higher than at day 14 (p -value = 0.005).

Discussion

IgG effector mechanisms are influenced by the attached Fc N-glycans. Here we studied changes in IgG Fc N-glycosylation upon vaccination of 10 Caucasian adults and 10 African children. To our knowledge, glycosylation of the constant region of human plasma IgG1 is restricted to N-glycosylation at Asn297, and no literature reports on O-glycosylation of the constant region are available. IgG Fc N-glycosylation profiles were determined using a recently described fast nanoLC-ESI-MS method, which allows accurate registration of tryptic IgG1, IgG2 and IgG4 Fc N-glycopeptides in

Table 3. Friedman test for the IgG1 glycosylation features of Caucasian adults.

Glycosylation feature	Medians						p -values*	
	Protein A			Focetria 09			Protein A	Focetria 09
	Day 0	Day 21	Day 56	Day 0	Day 21	Day 56		
Fucosylation	91.6	91.6	91.5	91.0	90.7	91.9	0.061	0.061
Bisecting N	16.2	15.8	16.2	17.6	12.2	12.5	0.150	< 0.001
Galactosylation	56.9	57.7	57.1	62.1	73.0	68.9	0.082	< 0.001
Sialylation	14.0	13.6	14.7	16.7	24.9	23.6	0.905	< 0.001
SA/Gal	12.6	12.6	13.4	13.5	17.4	17.2	0.670	< 0.001

*Friedman test p -value < 0.05 are considered to be significant and are highlighted in bold.

a single analysis.²¹⁷ The relative expression levels were determined for a total of 46 IgG Fc N-glycopeptides (Table 2) which were assigned unambiguously on the basis of literature knowledge of IgG N-glycosylation.^{3,4,9,10,79} From these data a set of IgG Fc N-glycosylation features, namely fucosylation, galactosylation, sialylation, SA/Gal and the level of bisecting N-acetylglucosamine were determined.²¹⁷

There were no vaccine related longitudinal changes in glycosylation of total IgG1, IgG2, and IgG4. We did detect glycosylation changes of vaccine-specific IgG1 in time. Active immunization with influenza or tetanus toxoid induced higher levels of galactosylation and sialylation and decreased the bisecting GlcNAc of antigen-directed IgG1. Interestingly, we observed an increase in the number of SA/Gal upon vaccination which might indicate a differential regulation of β 4-galactosyltransferase and sialyltransferase activities involved in IgG Fc N-glycosylation during biosynthesis in B-lymphocytes.⁵⁷ We did not observe significant changes in the level of fucosylation for total IgG and antigen-directed IgG1. No further changes in the glycosylation profiles were observed upon the second immunization of the Caucasians with influenza (day 21) which was possibly due to the large time difference (35 days) between the boost vaccination and sampling.

Our results are in contrast to the results of murine immunization studies: active immunization of specific pathogen free CBA/Ca mice with BSA causes a decrease in the galactosylation level for anti-BSA IgG.³⁴ In a murine serum nephritis model, immunization caused a drastic reduction of the IgG sialic acid content.³⁵ Upon repeated immunization with ovalbumin, increased levels of IgG fucosylation have been observed for male ICR mice.²¹¹ Specific pathogen free mice transferred from a sterile to a conventional environment showed an initial increase in the total IgG galactose content up to day 17 after which it decreased.³⁴ This galactosylation effect on the level of total IgG is similar to our observation of an initial galactosylation increase on vaccine-specific directed IgG1. However, specific pathogen free control mice remaining in the sterile environment revealed a similar galactosylation change, suggesting that the

Table 4. Friedman test for the IgG1 glycosylation features of African children.

Glycosylation feature	Medians									<i>p</i> -values*		
	Protein A			Begrivac 04/05			Tetanus					
	Day 0	Day 14	Day 28	Day 0	Day 14	Day 28	Day 0	Day 14	Day 28	Protein A	Begrivac	Tetanus
Fucosylation	95.1	94.8	94.8	94.2	93.8	93.0	93.1	94.7	94.7	0.497	0.741	0.135
Bisecting N	11.8	11.5	11.6	11.9	7.5	10.0	13.4	10.3	10.8	0.122	< 0.001	0.160
Galactosylation	47.5	49.0	48.2	56.0	74.3	67.9	51.3	64.7	62.1	0.027	< 0.001	0.006
Sialylation	15.1	16.1	15.3	20.1	30.4	25.9	16.6	26.5	24.4	0.122	< 0.001	0.060
SA/Gal	15.4	16.8	15.8	17.3	20.8	19.7	16.0	19.5	18.8	0.202	< 0.001	0.002

*Friedman test *p*-values < 0.05 are considered to be significant and are highlighted in bold.

observed effect was caused by aging of the mice rather than due to infection. Murine and human IgG subclasses/isotypes are different in various respects including their glycosylation, as murine IgG Fc N-glycans contain no bisecting GlcNAc and may carry N-glycolylneuraminic acid which is not found on human IgG.²¹⁸⁻²²⁰ In addition, lectins expressed on murine effector cells differ from those expressed in humans (e.g. SIGN-R1 an orthologue of the human DC-SIGN).^{47,48} Furthermore, murine glycoproteins and glycoproteins expressed in murine cell lines contain Gal α 1,3-Gal epitopes.²²¹⁻²²³ Hence, the study of specific glycosylation changes in murine models might not translate directly to the situation in humans.

IgG1 Fc N-glycans containing a bisecting N-acetylglucosamine have been shown to exhibit increased ADCC potency in *in-vitro* assays.^{213,214} The decrease in the level of bisecting N-acetylglucosamine on antigen-directed IgG1 upon vaccination might, therefore, suggest a lower ADCC potency of the anti-vaccine IgG1. While the high level of IgG1 Fc galactosylation found in our study is likewise expected to result in rather weak interactions with activating Fc γ -receptors and, consequently, ADCC,³⁵ high levels of Fc galactosylation have been found to lead to enhanced complement-dependent cytotoxicity (CDC).^{181,224} Tetanus toxoid²²⁵ and influenza envelope glycoprotein (hemagglutinin and neuraminidase)²²⁶⁻²²⁸ vaccines elicit high neutralizing antibody responses which have been correlated to vaccine-induced protective immunity. Influenza vaccine induced effector functions by non-neutralizing antibodies have also been shown to be involved in influenza clearance,²²⁹⁻²³³ and these effector functions are expected to be modulated by the glycosylation changes described here. The precise effector mechanisms involved in vaccine-mediated protection are far from clear and different mechanisms might apply for different viruses under different conditions.²³⁴

B-lymphocytes may produce IgGs with distinct Fc N-glycosylation profiles as is exemplified by the glycosylation differences between vaccine-specific IgG1 and total serum/plasma IgG1. First steps towards elucidating the underlying regulatory IgG glycosylation mechanisms in B-cells have recently been performed.⁵⁷ The glycosylation of IgG1 produced by B lymphocytes *in-vitro* is influenced by environmental factors (all-trans retinoic acid) and factors known to stimulate the innate (i.e. CpG oligodeoxynucleotide) or adaptive (i.e. interleukin 21) immune system.⁵⁷ Interestingly, the reported short-term increase in galactosylation and decrease in bisecting GlcNAc of IgG1 in oligodeoxynucleotide or interleukin 21 stimulated B-cells is in agreement with our observations during vaccination of humans. Modern vaccines such as those used in this study often contain adjuvants to enhance the immunogenicity of subunit (microbe strains and purified proteins) and DNA vaccines. Adjuvants can modify the outcome of epitope presentation to the immune system by specific T_H1 versus T_H2 polarization efficacy.²³⁵ The observed IgG Fc N-glycosylation changes, therefore, might be a result of a combined immune response towards the antigens and the adjuvant.

One may expect that prior to vaccination the individuals have had several encounters with cross reactive influenza strains via infections or previous vaccinations

resulting in the resting state IgG glycosylation profile at day 0. While glycosylation changes were observed within weeks after vaccination, IgG1 Fc glycosylation profiles obtained 9 months after influenza vaccination (determined for four African children, data not shown) were very similar to the profiles at day 0 and are likewise interpreted as resting state profiles.

Seasonal flu (influenza) vaccination usually precedes the encounter with the virus by weeks or months (prophylactic). Hence antibodies with high galactosylation and sialylation but with low incidence of bisecting GlcNAc are expected to be the ones involved in the defence against seasonal flu. Tetanus vaccination provides two scenarios as it is often performed directly after a wound (curative), and as preventive vaccination (prophylactic) which protects the individual 10-15 years. Our data indicate that depending on the vaccination time point the infectious agent will encounter IgGs with different glycosylation profiles (acute, high galactosylation, high sialylation versus resting state, low galactosylation, low sialylation) which might influence the antibody effector functions relevant in immunity. Interestingly, the observed switching points in the glycosylation features follow the antibody titers (data not shown).^{215,216}

In conclusion, analysis of different populations and races shed some light on natural effects of vaccination on antibody glycosylation profiles. Obviously, glycosylation patterns observed by us upon vaccination can not be easily explained from a teleological point of view, but it should be stressed that the regulatory aspects and functional implications of human IgG glycosylation features are still largely unknown, and that further research is required.

Acknowledgements

Maurice H.J. Selman thanks Hoffmann la Roche for financial support.

Supporting information

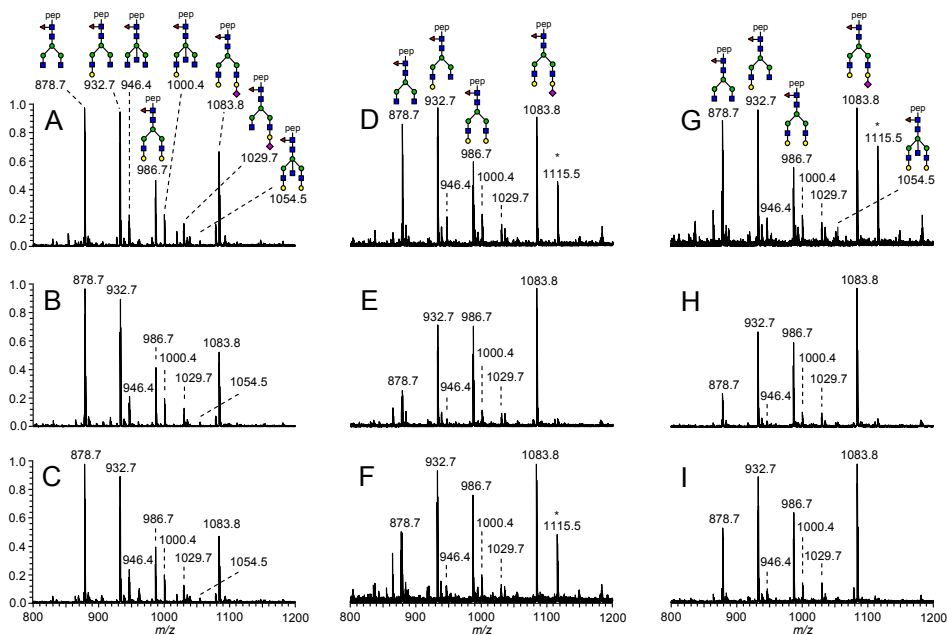


Figure S-1. Nano-RPLC-ESI-MS profiles (sum spectra of 1 min) of tryptic glycopeptides of IgG1 purified from African plasma by affinity chromatography with protein A (A-C), influenza vaccine (D-F) and tetanus toxoid (G-I) beads at day 0 (A, D, G), day 14 (B, E, H), and day 28 (C, F, I) after vaccination. *, Contaminant peak; glycan code as in Figure 1 of Chapter 1.

Table S-1. Comparison of IgG1 Fc N-glycosylation after protein A and antigen-specific purification.

Glycosylation feature	<i>p</i> -values*								
	Caucasians Focetria - Protein A			Africans Begrivac - Protein A			Africans Tetanus - Protein A		
	Day 0	Day 21	Day 56	Day 0	Day 14	Day 28	Day 0	Day 14	Day 28
Fucosylation	0.022	0.017	0.575	0.007	0.074	0.093	0.028	0.753	0.600
Galactosylation	0.005	0.005	0.005	0.005	0.005	0.005	0.028	0.028	0.028
Sialylation	0.022	0.005	0.005	0.005	0.005	0.009	0.046	0.028	0.028
SA/Gal	0.203	0.005	0.005	0.037	0.005	0.005	0.116	0.028	0.028
Bisecting GlcNAc	0.333	0.005	0.005	0.093	0.005	0.007	0.249	0.075	0.075
G0F ^a	0.005	0.005	0.005	0.005	0.005	0.005	0.028	0.028	0.028
G1F	0.009	0.005	0.005	0.114	0.005	0.007	0.028	0.046	0.075
G2F	0.007	0.005	0.005	0.059	0.005	0.005	0.345	0.028	0.028
G0FN	0.074	0.005	0.005	0.005	0.005	0.005	0.046	0.028	0.028
G1FN	0.203	0.005	0.005	0.013	0.013	0.203	0.463	0.116	0.173
G2FN	0.007	0.575	0.093	0.005	0.005	0.005	0.028	0.075	0.116
G1FS	0.285	0.007	0.009	0.169	0.005	0.022	0.116	0.249	0.116
G2FS	0.013	0.005	0.005	0.007	0.005	0.007	0.075	0.028	0.028
G1FNS	0.575	0.241	0.333	0.005	0.013	0.093	0.028	0.028	0.028
G2FNS	0.047	0.017	0.028	0.005	0.007	0.005	0.028	0.028	0.028
G0	0.059	0.241	0.139	0.575	0.028	0.005	0.249	0.345	0.046
G1	0.017	0.047	0.959	0.059	0.139	0.241	0.028	0.917	0.463
G2	0.005	0.013	0.093	0.114	0.028	0.037	0.028	0.173	0.463
G0N	0.799	0.093	0.028	0.646	0.007	0.114	0.345	0.917	0.345
G1N	0.646	0.009	0.017	0.386	0.017	0.059	0.075	0.917	0.753
G2N	0.005	0.007	0.114	0.007	0.017	0.022	0.028	0.028	0.046
G1S	0.575	0.169	0.093	0.005	0.114	0.203	0.116	0.345	0.028
G2S	0.037	0.013	0.074	0.022	0.093	0.093	0.028	0.173	0.075
G1NS	0.508	0.017	0.022	0.007	0.013	0.022	0.028	0.028	0.075
G2NS	0.386	0.005	0.059	0.017	0.017	0.028	0.028	0.028	0.046

*Wilcoxon Signed Ranks Test *p*-value < 0.013 are considered to be significant and are highlighted in bold.

^aglycan structural features are given in terms of number of galactoses (G0, G1, G2), fucose (F), bisecting N-acetylglucosamine (N), and N-acetylneuraminic acid (S).

Table S-2. Friedman test for IgG1 glycosylation features of European adults.

Glycosylation feature	Medians						<i>p</i> -values*	
	Protein A			Focetria 09				
	Day 0	Day 21	Day 56	Day 0	Day 21	Day 56	Protein A	Focetria 09
G0F ^a	15.3	15.4	14.8	12.1	6.4	9.4	0.273	< 0.001
G1F	32.0	31.6	31.4	28.4	23.1	23.6	0.007	< 0.001
G2F	17.4	18.1	17.4	18.873	26.7	24.0	< 0.001	< 0.001
G0FN	3.8	3.8	3.8	3.7	2.3	2.3	0.014	< 0.001
G1FN	8.4	8.2	8.3	8.8	5.9	6.3	0.301	< 0.001
G2FN	1.7	1.8	1.8	2.1	1.6	1.9	0.497	0.202
G1FS	1.8	1.8	1.8	1.8	1.5	1.6	0.082	0.007
G2FS	10.1	9.9	11.1	12.2	20.6	19.2	0.273	< 0.001
G1FNS	0.5	0.5	0.5	0.5	0.5	0.5	0.150	0.905
G2FNS	0.4	0.4	0.4	0.5	0.4	0.4	0.067	0.122
G0	1.0	0.9	1.0	1.3	0.7	0.7	0.003	0.067
G1	2.5	2.5	2.6	2.7	3.0	2.4	0.905	0.027
G2	1.9	1.9	2.0	2.3	2.7	1.9	0.497	0.014
G0N	0.3	0.3	0.3	0.3	0.2	0.2	0.014	0.741
G1N	0.6	0.6	0.6	0.7	0.4	0.4	0.273	0.122
G2N	0.3	0.3	0.3	0.4	0.4	0.4	0.150	0.273
G1S	0.2	0.2	0.2	0.2	0.2	0.2	0.067	0.670
G2S	0.8	0.8	0.8	0.9	1.2	0.8	0.273	0.008
G1NS	0.1	0.1	0.2	0.2	0.3	0.2	0.741	0.497
G2NS	0.2	0.1	0.2	0.2	0.2	0.2	0.061	0.301

*Friedman test *p*-values < 0.05 are considered to be significant and are highlighted in bold.

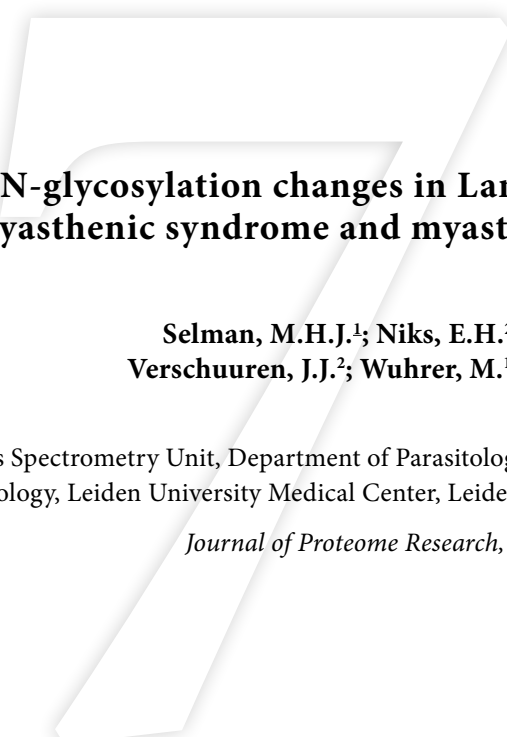
^aglycan structural features are given in terms of number of galactoses (G0, G1, G2), fucose (F), bisecting N-acetylglucosamine (N), and N-acetylneuraminic acid (S).

Table S-3. Friedman test for IgG1 glycosylation features of African children.

Glycosylation feature	Medians									<i>p</i> -values*		
	Protein A			Begrivac 04/05			Tetanus					
	Day 0	Day 14	Day 28	Day 0	Day 14	Day 28	Day 0	Day 14	Day 28	Protein A	Infuvac	Tetanus
G0F ^a	27.4	26.8	26.5	19.1	7.4	11.6	25.1	13.4	14.9	0.082	< 0.001	0.006
G1F	29.5	29.3	29.4	28.4	24.1	26.6	27.6	27.2	27.9	0.497	0.002	0.042
G2F	13.2	14.1	13.3	16.8	26.5	23.8	13.3	20.1	20.1	0.061	< 0.001	0.006
G0FN	5.1	4.7	5.0	4.1	1.4	2.5	4.9	2.5	2.7	0.007	< 0.001	0.006
G1FN	4.4	4.2	4.4	4.9	3.3	4.2	5.0	4.2	4.5	0.061	< 0.001	0.069
G2FN	0.7	0.8	0.7	1.0	1.1	1.1	1.1	1.2	1.1	0.741	0.407	0.846
G1FS	2.0	2.1	2.1	2.1	1.6	1.8	2.0	2.1	2.1	0.670	0.007	0.513
G2FS	11.1	12.0	11.1	14.9	26.6	20.8	11.9	21.4	20.0	0.061	< 0.001	0.006
G1FNS	0.3	0.3	0.3	0.4	0.4	0.4	0.4	0.5	0.5	0.497	0.741	0.311
G2FNS	0.2	0.2	0.2	0.4	0.3	0.3	0.3	0.4	0.4	0.202	0.273	0.846
G0	0.6	0.6	0.8	0.7	0.4	0.5	0.8	0.5	0.5	0.150	< 0.001	0.069
G1	1.5	1.4	1.4	1.6	1.9	2.0	1.9	1.5	1.4	0.301	0.407	0.115
G2	1.1	1.1	1.2	1.3	2.0	2.0	1.6	1.4	1.1	0.741	0.273	0.223
G0N	0.2	0.2	0.2	0.2	0.1	0.1	0.2	0.2	0.2	0.905	< 0.001	0.513
G1N	0.3	0.2	0.3	0.3	0.2	0.2	0.4	0.2	0.3	0.301	0.014	0.069
G2N	0.2	0.2	0.2	0.3	0.2	0.3	0.3	0.4	0.3	0.670	0.150	0.311
G1S	0.2	0.2	0.2	0.3	0.2	0.2	0.3	0.2	0.3	0.273	0.273	0.223
G2S	0.5	0.5	0.6	0.6	1.0	1.0	0.7	0.6	0.8	0.741	0.150	0.135
G1NS	0.1	0.1	0.1	0.2	0.2	0.2	0.2	0.2	0.2	0.273	0.122	0.311
G2NS	0.1	0.1	0.1	0.2	0.2	0.2	0.2	0.3	0.2	0.905	0.741	0.607

*Friedman test *p*-values < 0.05 are considered to be significant and are highlighted in bold.

^aglycan structural features are given in terms of number of galactoses (G0, G1, G2), fucose (F), bisecting N-acetylglucosamine (N), and N-acetylneuraminic acid (S).



IgG Fc N-glycosylation changes in Lambert-Eaton myasthenic syndrome and myasthenia gravis

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Abstract

N-glycosylation of the immunoglobulin Fc moiety influences its biological activity by e.g. modulating the interaction with Fcγ-receptors. Changes in IgG glycosylation have been found to be associated with various inflammatory diseases. Here we evaluated for the first time IgG Fc N-glycosylation changes in well-defined antibody-mediated autoimmune diseases, i.e. the neurological disorders Lambert-Eaton myasthenic syndrome, and myasthenia gravis with antibodies to muscle nicotinic acetylcholine receptors or muscle-specific kinase. IgGs were purified from serum or plasma by protein A affinity chromatography and digested with trypsin. Glycopeptides were purified and analyzed by MALDI-FTICR-MS. Glycoform distributions of both IgG1 and IgG2 were determined for 229 patients and 56 controls. We observed an overall age and sex dependency of IgG Fc N-glycosylation which was in accordance with literature. All three disease groups showed lower levels of IgG2 galactosylation compared to controls. In addition, LEMS patients showed lower IgG1 galactosylation. Notably, the galactosylation differences were not paralleled by a difference in IgG sialylation. Moreover, the level of IgG core-fucosylation and bisecting N-acetylglucosamine were evaluated. The control and disease groups revealed similar levels of IgG Fc core-fucosylation. Interestingly, LEMS patients below 50 years showed elevated levels of bisecting N-acetylglucosamine on IgG1 and IgG2, demonstrating for the first time the link of changes in the level of bisecting N-acetylglucosamine with disease.

Introduction

Immunoglobulins (Igs) represent an important part of the human immune system and can adapt to many different antigenic challenges. Structural variety of Igs is achieved by the expression of a multitude of hypervariable regions which create the antigen binding site and define the antigen specificity and affinity. Moreover, multiple classes (i.e. IgM, IgD, IgG, IgA, IgE) and subclasses (e.g. IgG1-4) of immunoglobulins are present in humans, differing in temporal expression patterns as well as in function. Igs are glycosylated, and the attached glycans have been implicated in modulating antibody function.^{19,35,43,44,146} IgG represents the most abundant antibody class in the human circulation, being present at a concentration of approximately 10 mg/ml.^{1,2} They consist of two heavy chains and two light chains. Parts of the heavy chains, together with the light chains, form two fragment antigen binding (Fab) moieties, whilst the remainders of the two heavy chains form the fragment crystallizable (Fc) moiety. A single biantennary complex-type N-glycan is attached to the asparagine residue at position 297 in the C_H2 domain of each heavy polypeptide chain of the Fc part. As a result of the glycosylation machinery a variety of biantennary complex-type N-glycans can be present (N-glycan microheterogeneity) which often carry a core-fucose and vary in the number of antenna galactose residues.^{3,4,147} Biantennary, core-fucosylated IgG Fc N-glycoforms with no, one, or two galactoses are referred to as G0F, G1F, and G2F, respectively. Moreover, these complex-type N-glycans may contain a bisecting N-acetylglucosamine and a sialic acid residue attached to an antenna galactose.

In healthy individuals IgG glycosylation features are reflected in age, gender and pregnancy.⁸⁻¹⁰ For both males and females it has been shown that the percentage of galactosylated complex-type N-glycans on IgG decrease with increasing age. Compared to males of similar age, young females (<30 years) were found to express higher levels of galactosylated species, and galactosylation showed a more pronounced decrease with increasing age for females than for males.¹⁰ During pregnancy an increase in IgG galactosylation is observed, reaching the highest levels towards the end of the gestation period. After delivery IgG galactosylation decreases to the levels observed before pregnancy.¹⁴

The biological activity of IgGs is modulated by Fc N-glycosylation. IgG sialylation has been shown to decrease in mice upon antigenic challenge, and it has been suggested that this change may provide a switch from innate anti-inflammatory activity in the steady state to an adaptive pro-inflammatory response.³⁵ Moreover, in a mouse arthritis model it has been shown that the anti-inflammatory properties of intravenous immunoglobulin depends on α 2,6-sialic acid linkages on IgG Fc N-glycans recognized by the C-type lectin SIGN R1 (an orthologue of the human DC-SIGN) expressed on murine macrophages in the splenic marginal zone.^{47,48} The lack of core-fucosylation of IgG1 Fc N-glycans has been shown to result in an enhanced binding to Fc γ -receptor IIIa expressed on macrophages leading to increased antibody-dependent

cellular cytotoxicity (ADCC).^{43,44} Enhanced ADCC activity of afucosylated IgG1 may have profound effects on the severity of antibody-mediated autoimmune diseases.⁴³⁻⁴⁵ Moreover, antibodies lacking core-fucosylation show enhanced efficacy in *in-vitro* ADCC assays and represent a potent new generation of anti-cancer therapeutics.^{44,46} Enhanced ADCC has also been related to an increased expression of bisecting N-acetylglucosamine on complex type Fc N-glycans.^{213,214} However, this was shown *in-vitro* and studies to confirm these findings *in-vivo* are required.

A decrease of IgG galactosylation has been shown to occur with various infectious diseases, autoimmune diseases, and cancer.^{3,32,147,236} IgGs with lowered galactosylation have been shown to exhibit increased potency in causing collagen-induced arthritis in a mouse model.¹⁹ Moreover, low degrees of IgG Fc galactosylation have been associated with high disease activity in rheumatoid arthritis.^{15,18,146}

The hitherto studied autoimmune diseases for which lowered IgG galactosylation has been demonstrated are characterized by both cellular and humoral autoimmunity. We here study IgG Fc N-glycosylation in autoimmune diseases, for which the role of pathogenic antibodies against well known target antigens has been described in detail. In the Lambert-Eaton myasthenic syndrome (LEMS) antibodies are directed against presynaptic voltage-gated calcium channels (VGCC), and in myasthenia gravis (MG) antibodies are found against either the postsynaptic muscle nicotinic acetylcholine receptors (AChR) or muscle-specific kinase (MuSK). The antibodies against these antigens impair the function of the neuromuscular junction resulting in fatigable weakness.²³⁷ Half of the patients with LEMS have a small-cell lung carcinoma²³⁸, which is known to express VGCC, while about 15% of the patients with MG have a thymoma.

Shifts in IgG Fc N-glycosylation features in well-defined antibody-mediated autoimmune diseases could provide valuable insights into which IgG Fc N-glycosylation features potentially influence antibody pathogenicity. In this study we, therefore, compared IgG Fc N-glycosylation features in LEMS and MG patients with presumably healthy controls by registering tryptic IgG1 and IgG2 glycopeptides with MALDI-FTICR-MS using a recently described protocol.¹⁴⁸ In addition, the effect of tumor on IgG Fc N-glycosylation was analyzed for LEMS patients.

Experimental section

Study cohort. The study cohort is described in Table 1. For the control group 27 human sera (15 male, 12 female) and 29 human citrate plasma samples (10 male, 19 female) were obtained from presumably healthy individuals. As no selection on the health status in the control cohort was performed, prevalence of diseases which are known to interfere with IgG Fc N-glycosylation is assumed to be low. Patients with LEMS with or without a small-cell lung carcinoma, non-thymoma AChR MG and MuSK MG were obtained from nationwide studies described previously.^{239,240}

Table 1. Human sample cohort

Group	Sex	NT ^a	T ^b	IS ^c	Youngest	Oldest	Mean age	STD age
Controls	Female	31			22	66	48.2	15.3
	Male	25			23	79	49.2	17.6
LEMS	Female	28	14	6	24	90	55.4	13.7
	Male	21	30	5	30	82	61.2	11.0
AChR MG	Female	64		19	11	84	40.8	17.7
	Male	34		7	33	90	51.2	20.4
MuSK MG	Female	27		16	15	86	52.3	18.5
	Male	11		8	18	79	63.5	15.7

^aNon-tumor^bTumor^cImmune suppression (i.e. prednisone, azathioprine or ciclosporin)

IgG glycopeptide profiling. IgG capturing, tryptic cleavage, glycopeptide purification, and MALDI-FTICR-MS analysis were performed as described elsewhere.¹⁴⁸ Briefly, human polyclonal IgGs were captured from 2 µl plasma or serum by affinity chromatography with Protein A-Sepharose™ Fast Flow beads (GE Healthcare, Uppsala, Sweden), and digested overnight at 37°C with 200 ng sequencing grade trypsin (Promega, Madison, WI). Glycopeptides were purified and desalted by hydrophilic interaction liquid chromatography (HILIC) solid phase extraction (SPE) using Sepharose® Cl-4B beads (GE Healthcare, Uppsala, Sweden). The entire procedure was performed in a 96-well plate format to allow high-throughput sample preparation. The purified and desalted IgG glycopeptides were spotted onto a polished stainless steel MALDI target plate (Bruker Daltonics, Bremen, Germany), overlaid with α-cyano-4-hydroxycinnamic acid matrix (CHCA, 5 mg/ml 50% ACN) and analyzed on an APEX-ultra 9.4 tesla FTICR mass spectrometer equipped with a intermediate pressure dual ESI/MALDI ion source (Apollo II), a Smartbeam™ laser system and a quadrupole mass filter (Bruker Daltonics). IgG glycopeptide profile spectra were generated by summation of 8 scans each performed after 9 ion accumulation cycles with 50 laser shots, effectively summing the ions of 3600 laser shots per spectrum. All data were acquired using Bruker ApexControl 3.0.0 software in expert mode. Mass spectra were internally calibrated using a list of known glycopeptides and Bruker DataAnalysis 3.4 in batch mode. Data processing was performed with the in-house developed software “Xtractor” (freely available at www.ms-utils.org/Xtractor) and Microsoft Excel. Relative intensities for twenty-six unambiguously assigned IgG Fc N-glycopeptides^{4,148} (15 IgG1 and 11 IgG2; Table 2) were obtained by integrating and summing four isotopic peaks followed by normalization to the total subclass-specific glycopeptide intensities. As part of the data processing, the level of non-detects within the dataset was determined. Non-detects were observed for some of the low abundant IgG Fc N-glycopeptides and

comprised less than 5% for each of these glycoforms (response variables). It is known that the bias generated by substitution of non-detects will be neglectable if less than 10% non-detects are present for each separate variable (i.e., glycoforms).^{241,242} Therefore, non-detects were substituted for the limit of detection (LOD) of the specific glycoform.

The level of galactosylation was calculated on the basis of the normalized intensities for the various Fc N-glycoforms according to the formula $(G1F + G1FN + G1FS + G1 + G1N + G1S) \times 0.5 + G2F + G2FN + G2FS + G2 + G2N + G2S$ for the IgG1 subclass and $(G1F + G1FN + G1FS + G1S) \times 0.5 + G2F + G2FN + G2FS$ for the IgG2 subclass. The prevalence of IgG sialylation was determined by summation of all sialylated Fc N-glycopeptide species (G1FS, G2FS, G1S and G2S for IgG1 and G1FS, G2FS, and G1S for IgG2). The level of bisecting N-acetylglucosamine was evaluated by summation of all bisected Fc N-glycopeptide species (G0FN, G1FN, G2FN, G0N, G1N, and G2N for the IgG1 subclass or G0FN, G1FN, G2FN, and G0N for the IgG2 subclass). The incidence of IgG1 and IgG2 N-acetylglucosamine is given as $(G0FN + G1FN + G2FN + G0N + G1N + G2N) / (G0F + G1F + G2F + G0 + G1 + G2 + G0FN + G1FN + G2FN + G0N +$

Table 2. Tryptic glycopeptides of human IgG subclasses 1 and 2.

Glycan species	IgG1 P01857 ^a	IgG2 P01859 ^a
No glycan	1189.5120	1157.5222
G0F ^d	2634.0459 ^b	2602.0561
G1F	2796.0987 ^b	2764.1089
G2F	2958.1515	2926.1617
G0FN	2837.1253 ^b	2805.1355
G1FN	2999.1781 ^b	2967.1883
G2FN	3161.2309	3129.2411
G1FS	3087.1941 ^b	3055.2043
G2FS	3249.2469	3217.2571
G0	2487.9880	2455.9982
G1	2650.0408	nd.*
G2	2812.0936	nd.*
G0N	2691.0673	2659.0775
G1N	2853.1202	nd.*
G2N	3015.1730	nd.*
G1S	nd.*	2909.1464
G2S	3103.1890	nd.*

^aSwissProt entry number

^bIsomeric glycopeptide species of IgG1 and IgG4.

*Not determined

^dglycan structural features are given in terms of number of galactoses (G0, G1, G2), fucose (F), bisecting N-acetylglucosamine (N), and N-acetylneuraminic acid (S).

G1N + G2N) and (G0FN + G1FN + G2FN + G0N) / (G0F + G1F + G2F + G0 + G0FN + G1FN + G2FN + G0N), respectively. The percentage of afucosylation is represented by summing the relative intensities of all non-fucosylated IgG Fc N-glycopeptide species (G0, G1, G2, G0N, G1N, G2N, G1S, and G2S for the IgG1 subclass and G0, G0N, and G1S for the IgG2 subclass).

Statistical analysis. The total subclass-specific normalized glycopeptide intensities were Log 10 transformed to obtain normally distributed IgG Fc N-glycosylation variables. Non-detects were included in the statistical analysis by substitution for the limit of detection (LOD). To determine possible associations two tailed Pearson and Spearman rank correlations were evaluated. Linear regression analysis was used to explore relationships between the response variables (IgG Fc N-glycopeptide values), and covariates (age and sex). Next, an interaction term between age and sex was included in the linear regression model. *p*-Values obtained by analysis of variance (ANOVA) were adjusted for multiple comparisons by Bonferroni correction, and *p*-values ≤ 0.05 were considered statistically significant. Data evaluation and statistical analysis were performed with Microsoft Excel and SPSS 16.0, respectively.

Results

IgG glycopeptide profiling by MALDI-FTICR-MS. In order to evaluate IgG Fc N-glycosylation changes in LEMS, AChR MG, and MuSK MG tryptic IgG Fc N-glycopeptides prepared from serum or plasma of control individuals and patients, were analyzed using a recently described MALDI-FTICR-MS method.¹⁴⁸ On the basis of published structural information on human IgG glycosylation^{3,4,9,10} the obtained high resolution mass spectra (an example is given in Figure 1) allowed unambiguous assignment of 15 IgG1 Fc N-glycopeptides and 11 IgG2 Fc N-glycopeptides (Table 2). Moreover, the integration and summation of four isotopic peaks for each assigned IgG Fc N-glycopeptide species provided very accurate relative quantification. Due to relatively low abundance of the signals and overlap with other signals, Fc N-glycopeptides of the human IgG4 subclass were not determined.

Age- and sex-dependency of IgG Fc N-glycosylation. To determine the effect of age and gender on IgG galactosylation within our cohort, we graphically evaluated IgG Fc galactosylation as a function of age. For all groups (controls, LEMS, AChR MG, and MuSK MG) the relative abundances of agalactosylated IgG species increased at higher age, as is represented for the G0F and G0FN species of IgG1 (Figure 2A and C) and IgG2 (Figure S-1A and D) of control and LEMS individuals. On the contrary, glycoforms exhibiting two galactoses decreased with age, which is shown for the G2F and G2 species of IgG1 (Figure 2B and D) and for the G2F glycoform of IgG2 (Figure S-1C). Interestingly, the IgG2 G1F glycoform (Figure S-1B) showed an age-dependency which was similar to that of the IgG2 G2F glycoform.

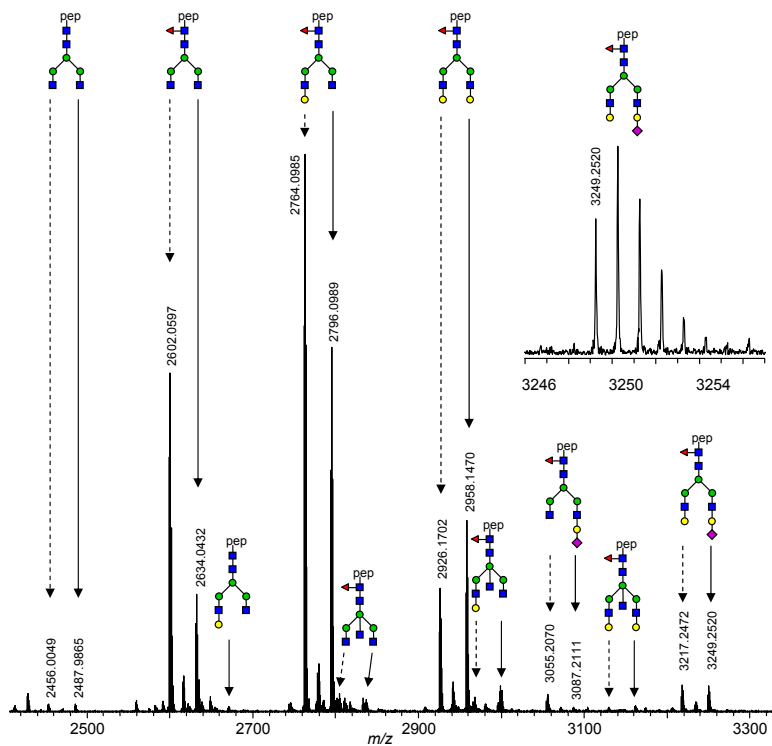


Figure 1. MALDI-FTICR-MS spectrum of tryptic IgG Fc N-glycopeptides. Continued arrows, IgG1 glycopeptides; dashed arrows, IgG2 glycopeptides; glycan code as in Figure 1 of Chapter 1.

In control individuals as well as LEMS, AChR MG and MuSK MG patients, differences in IgG galactosylation were observed between females and males, with the younger females (< 30 years) showing higher levels of IgG galactosylation than males of the same age (data not shown). Moreover, linear regression analysis of IgG galactosylation with age showed for females within each group a steeper drop (p -value < 0.01) in the level of IgG galactosylation with increasing age than for males, reaching similar levels as males between the ages of 60 to 70 years.

Next to changes in galactosylation we evaluated age and sex dependent variations in IgG Fc sialylation, incidence of bisecting N-acetylglucosamine and core-fucosylation. The G2F glycan structure represents the substrate of the sialyltransferase reaction which results in G2FS. For the controls and the three disease groups our data revealed a G2FS age and sex dependency similar to that observed for the G2F species (data not shown). Linear regression analysis of the total level of IgG sialylation with age showed a decrease in IgG sialylation with increasing age for each group. Moreover, younger females showed higher levels of IgG sialylation than males of similar age. For females

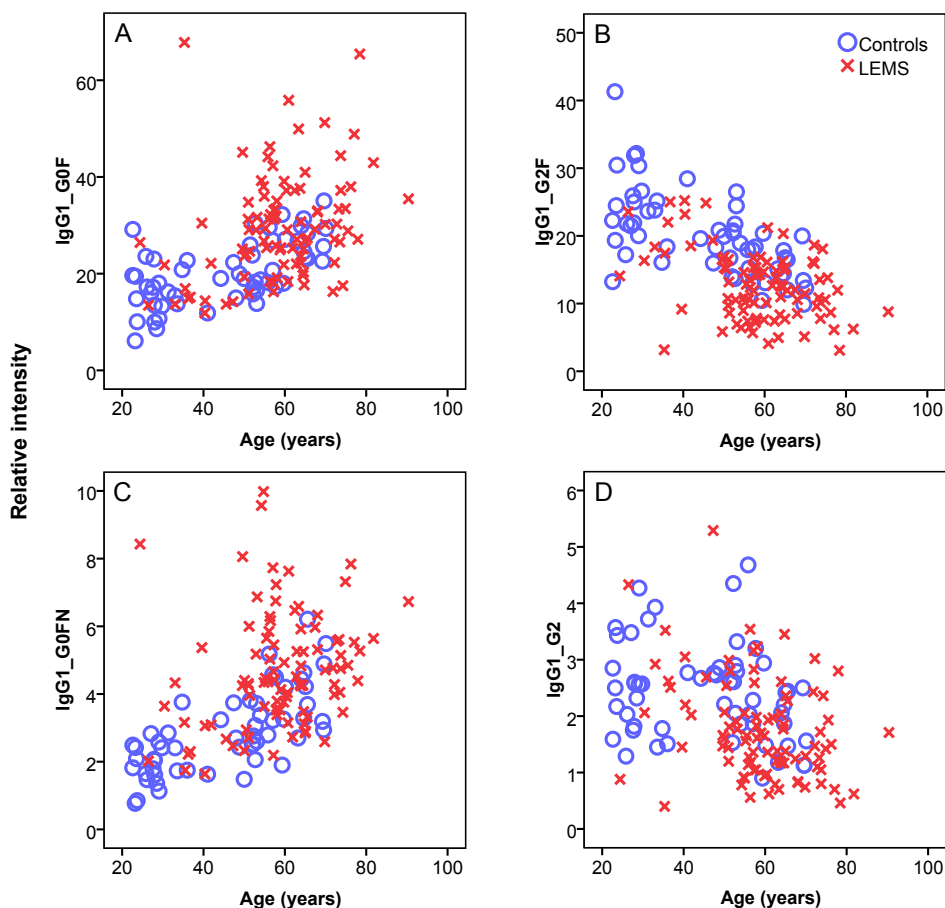


Figure 2. Age dependency of IgG1 Fc galactosylation. Tryptic IgG glycopeptides were desalted and purified by Sepharose SPE, spotted on a stainless steel MALDI target plate, overlaid with CHCA, and analyzed by MALDI-FTICR-MS. Signal intensities of individual glycoforms were normalized to the total subclass-specific glycopeptide intensities. Circles represent IgG1 Fc N-glycopeptides of control individuals, and crosses represent IgG1 Fc N-glycopeptides of LEMS patients.

a more pronounced decrease in IgG sialylation levels with increasing age was observed than for males (p -value < 0.01), reaching similar levels as males between the age 60 to 70 years.

The incidence of bisecting N-acetylglucosamine species increased at higher age for the control group and the AChR MG and MuSK MG patients, as shown for IgG1 (Figure 3) and IgG2 (Figure S-2). Within all groups (control, LEMS, AChR MG and MuSK MG), no sex-related variations on the levels of bisecting N-acetylglucosamine were observed.

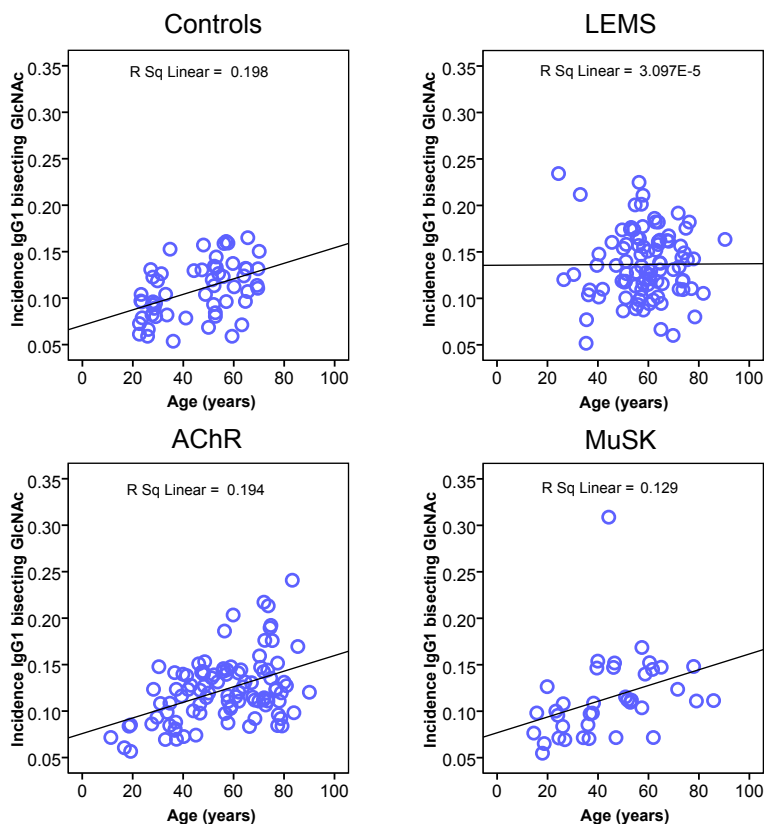


Figure 3. Age-related changes in the incidence of IgG1 Fc bisecting N-acetylglucosamine in control individuals, and patients suffering from LEMS, AChR MG or MuSK MG.

Linear regression analysis of the level of IgG1 and IgG2 Fc core-fucosylation with age revealed a significant age effect for MG patients producing MuSK autoantibodies (p -value < 0.01). Although controls as well as LEMS and AChR MG patients showed a trend towards increasing levels of non-fucosylated species at higher age this was not statistically significant. Within the cohort, no clear sex effects were observed for the fucosylated glycopeptide species.

Statistical analysis of disease-associated IgG Fc N-glycosylation features. Because of the age- and sex-dependency of various IgG glycosylation features observed in our cohort (see above) as well as by others,⁸⁻¹⁰ correction for these confounding factors was performed to allow statistical comparison of IgG Fc N-glycosylation features from controls, LEMS, AChR MG and MuSK MG by linear regression. Models generated by regression analysis, however, may be biased or misleading if the variables are not normally distributed. Therefore, we evaluated the distribution of the relative abundances

for each IgG Fc N-glycopeptide within the separate groups prior to statistical data analysis. This revealed a skewed distribution for most IgG Fc N-glycopeptides, which could be restored to normality by Log 10 transformation. The Log 10 transformed relative abundances were corrected for age, sex and the interaction between age and sex using linear regression analysis.

Pair wise comparison (post-hoc analysis) of IgG Fc N-glycosylation for controls, LEMS, AChR MG and MuSK MG was performed for the specific glycosylation features galactosylation, level of bisecting N-acetylglucosamine, sialylation, and afucosylation (Figure 4A; Figure S-3A) as well as for the individual glycoforms (Figure 4B and C; Figure S-3B and C). This revealed that IgG1 and IgG2 of LEMS patients had a lower degree of galactosylation compared to IgGs of controls (p -value < 0.01). Moreover, higher levels of agalactosylated IgG1 (G0F, G0FN, G0 and G0N) and IgG2 (G0F, G0FN and G0N) species (p -value < 0.01) were observed in the LEMS patients. On the contrary, several of the digalactosylated glycoforms were decreased. Notably, the lowered IgG1 and IgG2 Fc galactosylation of LEMS patients was already indicated by the data presented in Figure 2 and Figure S-2, which over the whole age range showed a tendency towards lower IgG1 and IgG2 Fc galactosylation of LEMS patients compared to control individuals.

While LEMS patients compared to the control individuals showed a lower degree of galactosylation for both IgG1 and IgG2, galactosylation in AChR MG and MuSK MG patients was only lowered for IgG2 (p -value < 0.05; Figure S-3A). At the level of individual IgG2 glycoforms, the G0F species were increased, whilst both G1F and G2F were lowered for all disease groups. As already mentioned previously, the acceptor structure for the sialyltransferase reaction resulting in G2FS is represented by the G2F glycan structure. Although diseased individuals expressed lower G2F species compared to control individuals, no differences were observed for the level of IgG1 and IgG2 Fc sialylation. At the individual IgG glycoform level, however, higher prevalence of IgG1 G1FS and IgG2 G1FS species (p -value < 0.05) were observed for the LEMS and AChR MG patients compared to the control individuals. Moreover, IgG1 G2FS was lowered in LEMS patients compared to the control individuals. For the MuSK MG patients only the IgG2 G1FS species was higher (p -value < 0.05) than for the control group. As no overall change in sialylation was observed (Figure 4A and Figure S-3A), the differences in G1FS levels are considered to predominantly reflect changes in galactosylation rather than specific sialylation changes.

A higher level of IgG1 and IgG2 Fc bisected N-acetylglucosamine (p -value < 0.01) was observed for the LEMS patients in comparison to the controls. Evaluation of the individual bisected glycopeptides revealed a higher prevalence of the G0FN and G0N species for IgG1 as well as IgG2 (p -value < 0.01). LEMS patients tended to have elevated levels of bisecting N-acetylglucosamine already at younger age, which remained at the same level over the whole age range. Yamada *et al.* described that the incidence of bisecting N-acetylglucosamine in healthy individuals increased until the age of

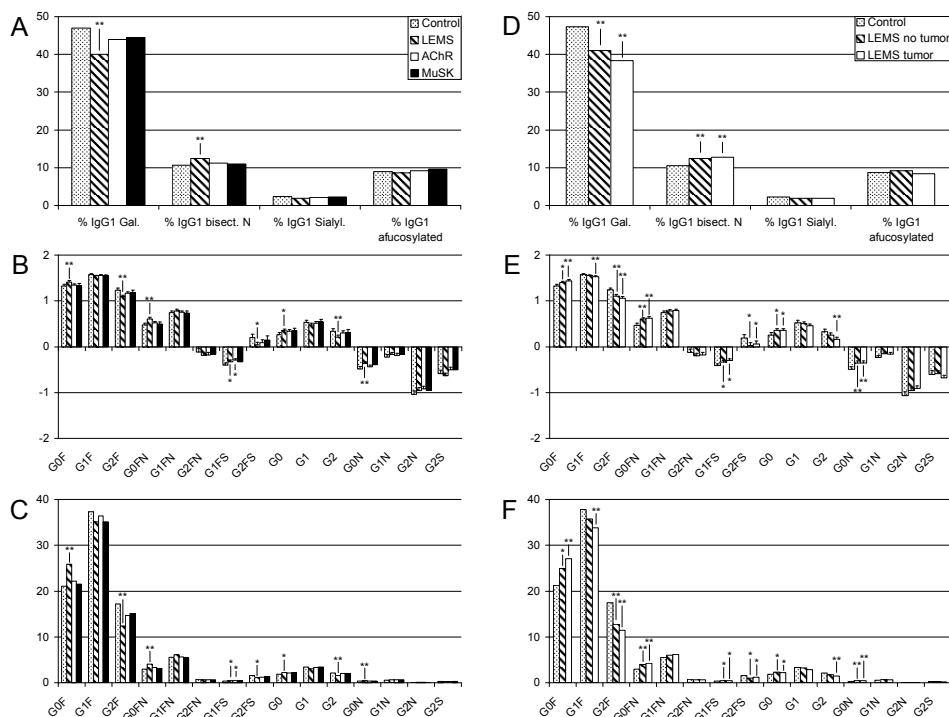


Figure 4. Statistical comparison of IgG1 Fc N-glycosylation for control and diseased individuals. A and D represent the statistical comparison of different IgG1 Fc N-glycosylation features, while B, C, E and F represent the statistical comparison of the individual IgG1 Fc N-glycopeptides. *, Significant from controls with p -value < 0.05 ; **, Significant from controls with p -value < 0.01 .

50 years at which it reached a plateau.⁹ Therefore, we divided the LEMS patients and control individuals in two groups; i.e. younger than 50 years and older than 50 years, and reevaluated the level of bisecting N-acetylglucosamine within these age groups. This revealed a higher incidence of IgG1 and IgG2 bisecting N-acetylglucosamine level (p -value < 0.01) for LEMS patients younger than 50 years compared with control individuals of similar age and gender. Notably, female LEMS patients below 50 years showed a higher level of IgG2 bisecting N-acetylglucosamine than male LEMS patients of similar age (p -value < 0.05). No statistically significant differences were observed for the level of bisected N-acetylglucosamine between LEMS patients and control individuals above 50 years. Evaluation of the individual bisecting N-acetylglucosamine species revealed a higher prevalence for the IgG1 and IgG2 G0FN species in LEMS patients younger than 50 years compared with age matched control individuals (p -value < 0.01). By contrast, LEMS patients older than 50 years showed a higher prevalence of the IgG1 G0N and G2N species compared with the age matched control individuals (p -value < 0.05).

Statistical comparison of the total level of IgG1 Fc afucosylation revealed no difference between controls and diseased (LEMS, AChR MG and MuSK MG) for IgG1. Moreover, AChR MG and MuSK MG patients showed levels of IgG2 afucosylation which were similar to those of controls. By contrast, higher levels of IgG2 afucosylation were observed for LEMS patients (p -value < 0.05). Evaluation at the individual IgG glycoform level revealed that only for the LEMS patients some non-fucosylated species (IgG1 G0, IgG1 G0N, and IgG2 G0N) differed statistically significant from the controls.

Analysis of the role of confounding factors such as tumors and immune suppression. To determine potential tumor-related IgG Fc N-glycosylation differences, LEMS patients with and without tumor were compared with the controls group (Figure 4D-F and Figure S-3D-F). The overall IgG1 and IgG2 Fc N-glycosylation of both LEMS groups (tumor, non-tumor) was comparable to the IgG Fc N-glycosylation observed for LEMS patients prior splitting. In addition to the IgG Fc N-glycosylation features already described in the previous sections, LEMS patients with a tumor showed a higher level of IgG2 sialylation compared to the controls (p -values < 0.05) and compared to LEMS patients without tumor (p -values < 0.05). No significant changes were observed for the degrees of IgG1 sialylation in LEMS tumor versus controls and versus LEMS non-tumor.

Next to tumor-related IgG Fc N-glycosylation differences, we evaluated potential associations of IgG Fc N-glycosylation with the use of oral immune suppressants (i.e. prednisone, azathioprine or ciclosporin). The use of immune suppressants could not be associated with changes in IgG Fc N-glycosylation features.

Discussion

IgG glycosylation changes have been shown to occur in various infectious diseases, autoimmune diseases, and cancer.^{3,15,18,32,146,147} Here we studied IgG Fc N-glycosylation in the context of well-defined autoantibody-mediated autoimmune diseases. Our results show that LEMS and MG subtypes are associated with changes in IgG1 and IgG2 Fc N-glycosylation (e.g. decrease in galactosylation). Experiments were performed using a recently described high-throughput IgG glycosylation profiling method which registers IgG1 and IgG2 tryptic Fc N-glycopeptides by MALDI-FTICR-MS.¹⁴⁸ The analyzed cohort comprised a total of 285 samples from four groups, namely (1) presumably healthy control individuals, (2) patients with LEMS, (3) non-thymoma MG patients with autoantibodies directed against the muscle nicotinic acetylcholine receptor and (4) MG patients with autoantibodies against muscle-specific kinase. Information was obtained on the relative expression levels of 15 IgG1 Fc N-glycan structures and 11 IgG2 Fc N-glycan structures. In order to give a simplified and informative overview on the IgG Fc N-glycosylation status, next to the expression levels of the individual glycoforms, the overall incidence of the glycosylation features galactosylation, sialylation, fucosylation, and level of bisecting N-acetylglucosamine were determined (Figure 4).

IgG glycosylation profiles are known to be influenced by age and gender.⁸⁻¹⁰ Our results showed in all groups a higher degree of IgG1 and IgG2 galactosylation for young females (<30 years) than for males of similar age, which is in agreement with previous findings.¹⁰ We observed a decrease of IgG1 and IgG2 Fc galactosylation with age which is in accordance with literature reports on age dependent changes of the glycosylation of total human IgG.⁸⁻¹⁰ This decrease was more pronounced in females than in males. Above age 60 years an equally low plateau of low levels of IgG1 and IgG2 galactosylation and sialylation was reached in males and females. In contrast to literature data, which are mostly based on the combined analysis of Fc and Fab N-glycosylation of total IgG, our results reflect the specific Fc N-glycosylation analysis of IgG1 and IgG2 subclasses.

IgG Fc N-glycosylation results obtained for the four groups (controls, LEMS, AChR MG, and MuSK MG) were compared after log-transformation and correction for age, sex, and the interaction of age and sex giving the following findings: IgG2 galactosylation was found to be lower in all three disease groups than in controls. IgG1 galactosylation was found to be significantly lowered in LEMS patients only, while the other two disease groups showed a similar trend. Notably, no difference in the overall degree of IgG1 and IgG2 sialylation was found. Thus, the observed differences in IgG galactosylation between the controls and the disease groups are not paralleled by a difference in IgG sialylation. This indicates that different factors are influencing IgG galactosylation and sialylation in different ways: while age-associated decrease of IgG galactosylation and sialylation appear to go in parallel, the disease-associated decrease in IgG galactosylation is not paralleled by a sialylation decrease. This might reflect changes in the regulation of the involved glycosyl transferases which could be assumed to be considerably different in ageing and in disease. Another indication for the distinct regulation of IgG galactosylation and sialylation has been reported by Kaneko *et al.* (2006), who described that antigenic challenge leads to a specific change in IgG sialylation in mice, which was not reflected in a corresponding change in IgG galactosylation.³⁵ Hence, comprehensive analysis of IgG Fc N-glycosylation for mice as well as humans should involve registration of both galactosylation and sialylation levels.

The only partially linked regulation of IgG sialylation and galactosylation is paralleled by presumably distinct functions of these two IgG glycosylation features: IgG Fc sialylation has been indicated as an anti-inflammatory signal which acts via the receptor SIGN-R1 (an orthologue of the human lectin DC-SIGN) on murine macrophages.^{35,48} The degree of IgG galactosylation has been reported to influence the pathogenicity of IgGs.¹⁹ In a murine collagen-induced arthritis model anti-collagen IgG which had been treated with β -galactosidase (resulting in elevated levels of IgG G0F) was found to be more potent in inducing arthritis than non-treated control IgG. Moreover, agalactosylated IgG has been shown to have a higher efficacy in complement activation than galactosylated IgG, though the *in-vivo* relevance of this finding has not been demonstrated.^{20,243}

Changes in overall IgG galactosylation have been reported for many other autoimmune and inflammatory diseases. Rheumatoid arthritis and osteoarthritis have been reported to result in an increase of the level of agalactosylated IgG N-glycans of approximately 25% and 10%, respectively: rheumatoid arthritis patients showed an average of 50% agalactosylated IgG, which was 35% for osteoarthritis patients and 25% for control individuals.³ These data were obtained by analyzing released N-glycans of total serum IgG and, therefore, refer to the combined Fc and Fab glycosylation of all IgG subclasses. Fab glycosylation contributes approximately 20% to the overall IgG N-glycosylation profile and shows only minor changes in rheumatoid arthritis.²⁴⁴ By contrast, Fc N-glycosylation of all IgG subclasses is known to be affected by rheumatoid arthritis.⁴ As both changes in total IgG N-glycosylation profiles (Fab and Fc) in rheumatoid arthritis will predominantly reflect Fc N-glycosylation changes, it seems legitimate to us to compare combined Fab and Fc N-glycosylation data from literature with the Fc N-glycosylation results we obtained here.

The total level of agalactosylated IgG1 and IgG2 Fc N-glycans (sum of G0F, G0, G0FN, and G0N) found in our study was 33% (IgG1) and 44% (IgG2) for LEMS while in controls this was 26% (IgG1) and 37% (IgG2). Thus, for both IgG1 and IgG2 the LEMS-associated decrease in Fc galactosylation was 7%. For AChR MG and MuSK MG the disease-associated decrease in IgG2 Fc galactosylation was approximately 4% and 5%, respectively. Clearly, the galactosylation changes associated with LEMS and MG are less pronounced than those described for arthritis and osteoarthritis. While the immunological relevance of the observed IgG galactosylation changes in LEMS and MG is still unclear, a decrease of IgG Fc galactosylation is generally leading to a less efficient complement activation by IgG.¹⁸¹ Moreover, lowered IgG Fc galactosylation enhanced the potency of IgG autoantibodies in a murine arthritis model¹⁹ and has been shown to correlate with high disease activity and progressive disease for rheumatoid arthritis.^{15,18}

Next to galactosylation and sialylation, we monitored the incidence of bisecting N-acetylglucosamine as well as the degree of core-fucosylation of IgG Fc N-glycans. The incidence of IgG1 and IgG2 bisecting N-acetylglucosamine was found to increase with age for controls, AChR MG, and MuSK MG, which is in accordance with previous findings for total IgG of healthy individuals.⁸⁻¹⁰ No difference was found in the levels of bisecting N-acetylglucosamine between controls, AChR MG, and MuSK MG. Interestingly, LEMS patients of age below 50 years showed elevated levels of bisecting N-acetylglucosamine on both IgG1 and IgG2, while these levels were not elevated for LEMS patients of age above 50 years. Elevated levels of bisecting N-acetylglucosamine have been associated with increased ADCC.²¹³ However, *in-vivo* relevance has not been shown yet. To the best of our knowledge, our study for the first time shows the link of changes in the level of bisecting N-acetylglucosamine with disease.

Human IgGs from various body fluids has consistently been found to exhibit a low degree of non-fucosylated Fc N-glycans,¹⁴⁶ which is in accordance with our

findings. Increasing levels of non-fucosylated species at higher age were observed for MuSK MG patients (p -value < 0.01), while controls, LEMS and AChR MG patients only showed a similar trend. In juvenile chronic arthritis and rheumatoid arthritis, a disease-associated increase in IgG Fc fucosylation has been observed.^{23,25} By contrast, we observed a small decrease in the core-fucosylation of IgG2 Fc N-glycans of LEMS patients, while no disease-associated changes were observed for AChR MG and MuSK MG patients.

In approximately half of the LEMS group a small-cell lung carcinoma was present, which is strongly associated with the development of the disease.^{237,238} Interestingly, all LEMS patients revealed similar IgG Fc N-glycosylation profiles independent of the presence of a tumor.

Pathogenic autoantibodies in AChR MG are mainly of the IgG1 subclass and trigger complement mediated damage to the neuromuscular synapse.²⁴⁵ By contrast, disease severity in MUSK MG appears to be related to autoantibodies of the IgG4 subclass.²⁴⁶ Remissions and exacerbations in these autoimmune disorders are related to autoantibody titers in individual patients.²⁴⁷ However, the inter-individual relationship is weak meaning that patients can be severely affected while having very low autoantibody titers and vice versa. Besides the autoantibody titers, differences in IgG Fc N-glycosylation may also be related to the fluctuating muscle weakness. For MUSK MG and AChR MG, we did not find a correlation between disease severity scores (DSS) and IgG Fc N-glycosylation, while DSS were not available for the LEMS patients. As an alternative attempt to correlate clinical disease severity with overall IgG Fc N-glycosylation, longitudinal studies (repeated observations of the same patients over long periods of time) should be performed. Notably, the overall IgG Fc N-glycosylation as studied here may not reflect the characteristics of antigen-specific IgG.^{22,24} Future studies of autoantigen-specific IgG Fc N-glycosylation will possibly reveal associations between disease severity and glycosylation features.

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Supporting information

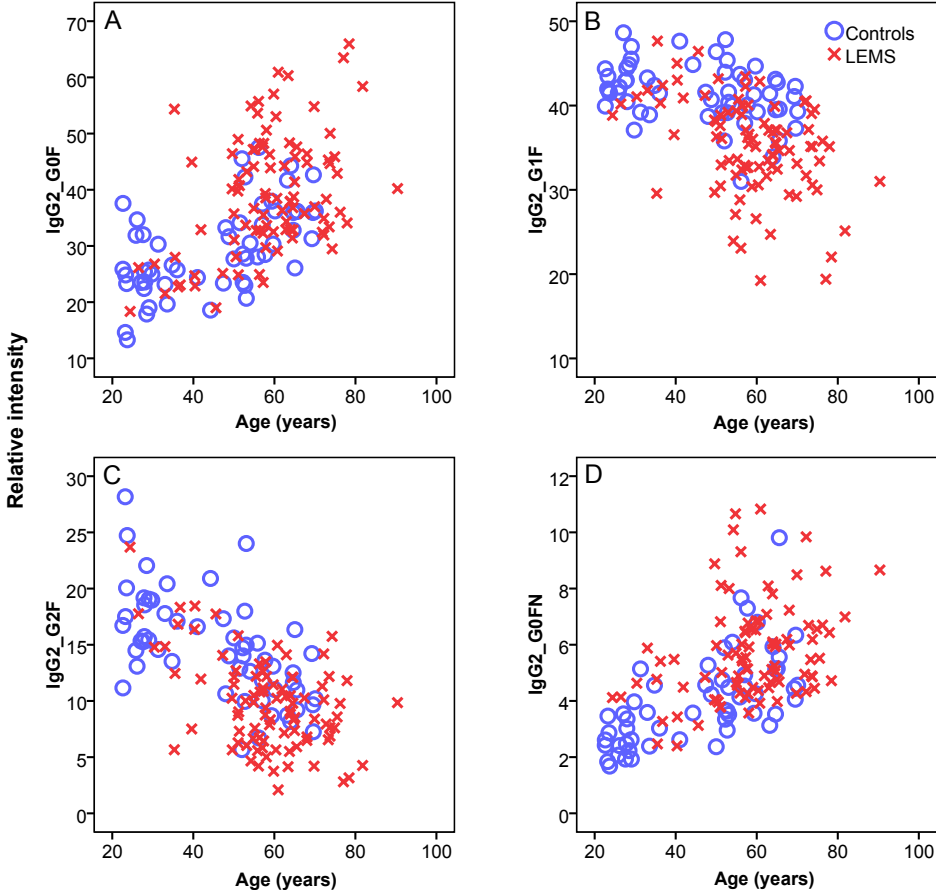


Figure S-1. Age dependency of IgG2 Fc galactosylation. Tryptic IgG glycopeptides were desalted and purified by Sepharose SPE, spotted on a stainless steel MALDI target plate, overlaid with CHCA, and analyzed by MALDI-FTICR-MS. Signal intensities of individual glycoforms were normalized to the total subclass-specific glycopeptide intensities. Circles represent IgG2 Fc N-glycopeptides of control individuals, and crosses represent IgG2 Fc N-glycopeptides of LEMS patients.

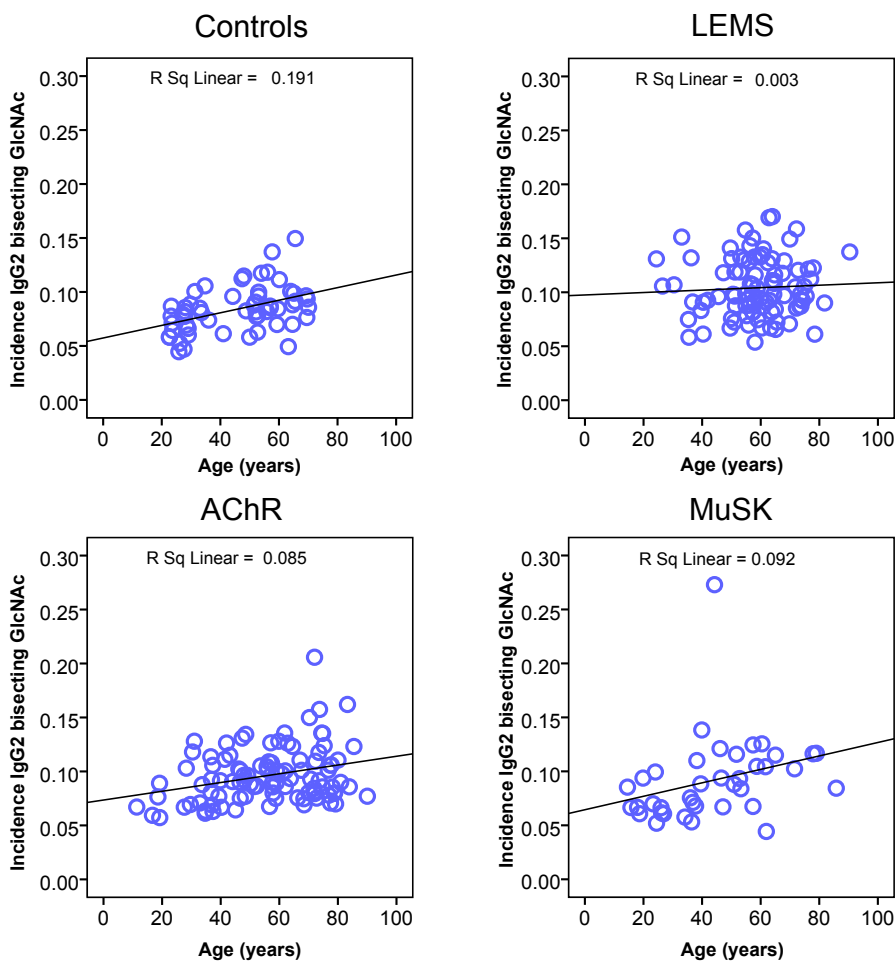


Figure S-2. Age-related changes in the incidence of IgG2 Fc bisecting N-acetylglucosamine in control individuals, and patients suffering from LEMS, AChR MG or MuSK MG.

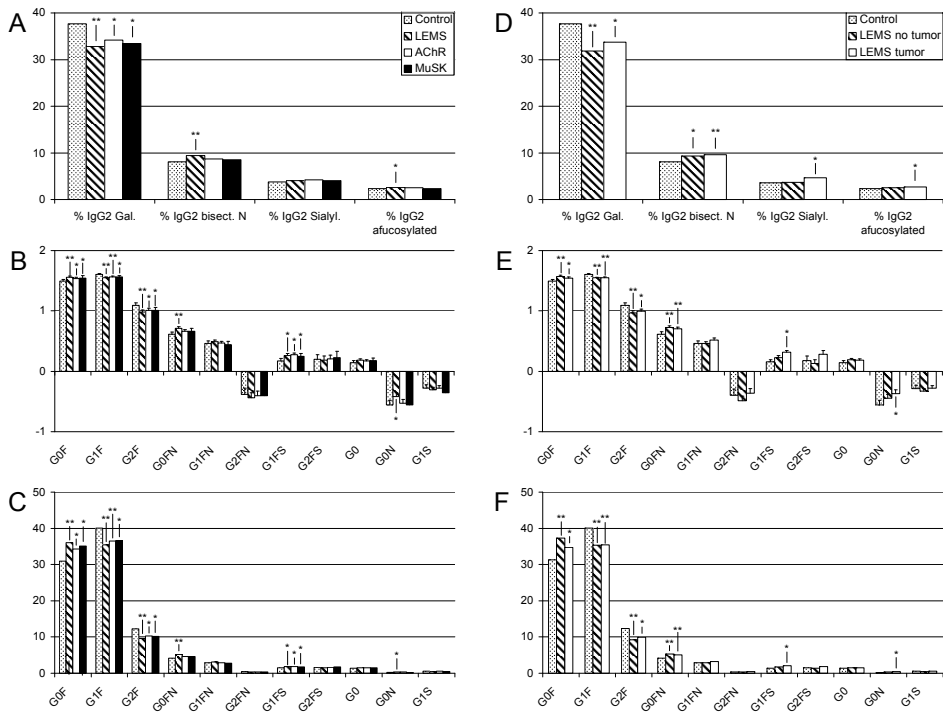
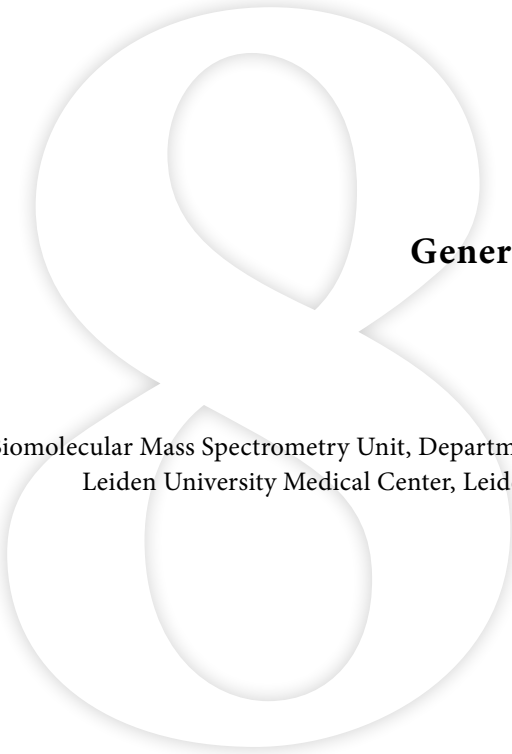


Figure S-3. Statistical comparison of IgG2 Fc N-glycosylation for control and diseased individuals. A and D represent the statistical comparison of different IgG2 Fc N-glycosylation features, while B, C, E and F represent the statistical comparison of the individual IgG1 Fc N-glycopeptides. *, Significant from controls with p -value < 0.05 ; **, Significant from controls with p -value < 0.01 .



General discussion

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Over the last decade the number of approved biopharmaceuticals harboring glycosylation has been rapidly growing. Glycans attached to therapeutic proteins affect their physical properties, stability and efficacy, and aberrant glycoforms may cause serious adverse effects. Detailed characterization of protein glycosylation is of utmost importance in pharmaceutical industry in order to maintain product quality and safety. Therapeutic immunoglobulins received much attention over the last years due to drastic enhancement of ADCC observed for antibodies lacking the core-fucoses on the Fc N-glycan.^{43,44} Therefore, IgG Fc N-glycosylation of biotechnologically produced IgGs is being designed in order to maximize the efficacy in e.g. anti-cancer therapy (next generation therapeutic antibodies).^{5,46}

Glycan structural and linkage information of human IgG Fc N-glycans is well characterized and has been shown to be influenced by age, sex, pregnancy and health state.^{8-10,147} For a variety of infectious and autoimmune diseases, and cancer aberrant glycosylation has been reported.^{3,21,30-32} A common observation for many of these diseases is the lowered IgG Fc galactosylation and sialylation.¹⁴⁷ Fc N-glycans have been shown to influence the biological activity of IgG by e.g. modulating Fcγ-receptor binding.^{19,35,38,39,43,44,146} Sensitive and detailed characterization of aberrant IgG Fc N-glycosylation profiles could provide valuable information on the regulatory mechanisms involved in a broad range of antibody mediated diseases. By implementing this knowledge into sensitive enzyme-linked immunosorbent assays capable of discriminating IgG with different glycosylation (e.g. IgG with various levels of galactosylation but lacking the core-fucose), it might become possible to discriminate pathogenic from non-pathogenic situations. This would open the door for treating potentially harmful changes in glycosylation such as the decreased levels of core-fucosylation observed during pregnancy on IgG1 alloantibodies against the human platelet antigens of the fetus (causing fetoneonatal alloimmune thrombocytopenia)⁴⁵ already in an early stage prior to the actual harm/damage.

Clearly, in view of the above mentioned interest in antibody glycosylation in the biopharmaceutical as well as in the clinical setting, analytical methods are needed to study these glycans. Detailed analysis of protein glycosylation is a challenging and time-consuming task due to the large structural variety in the attached glycans even for a single glycosylation site (microheterogeneity). In this thesis novel approaches for fast, miniaturized and high-throughput analysis of IgG Fc N-glycosylation are presented, and the utility of these methods has been demonstrated for clinically relevant research questions. Due to the complexity of human polyclonal IgG, Fc N-glycopeptide MS profiles might contain glycan information from a mixture of IgG subclasses. This is especially occurring with MALDI-MS approaches while obviously for LC-MS approaches it is a much less relevant issue. When analyzing biotechnologically produced therapeutic monoclonal antibodies, MALDI-MS and LC-MS both provide similar IgG Fc N-glycosylation information but differ in analysis speed. When analyzing monoclonal antibodies MALDI-MS offers the highest potential

for accurate high-throughput IgG Fc N-glycosylation analysis. By contrast, LC-MS is better suited for accurate IgG Fc N-glycosylation analysis in complex biological samples such as plasma and serum. In general, LC-MS is less prone to in-source and metastable decay of highly sialylated glycopeptides than MALDI-MS. Nevertheless, the labile nature of glycoconjugates under standard ESI-MS proteomics settings requires several optimizations. Decay of glycoconjugates commonly occurs in the source and during the ion-transfer. Thus lowering of the potential differences during ESI (sprayer and MS inlet) and the ion-transfer (capillary and skimmer or lenses) is a good start to reduce glycan decay. Which potential differences have to be lowered, however, strongly depend on the type of mass spectrometer.

The IgG glycosylation analysis methods described in **Chapter 2** and **Chapter 4** of this thesis are robust, can handle large sample cohorts, reveal glycosylation features such as galactosylation, sialylation, fucosylation, and the incidence of bisecting N-acetylglucosamine at an IgG subclass-specific level, and can be applied to analyze the Fc N-glycosylation of recombinantly produced IgG in great detail. Fast or high-throughput analysis was achieved by optimizing the entire analytical process, including sample preparation, glycopeptide detection and data processing. These techniques were then successfully applied for specific clinical research questions where IgG Fc N-glycosylation is believed to play a role (e.g. vaccination, autoimmune diseases). Other applications that one may envision involve longitudinal studies where total and autoantigen-specific IgG Fc N-glycosylation changes are evaluated to reveal associations between glycosylation features and e.g. disease severity. Furthermore, these sensitive fast profiling techniques are expected to prove their use for monitoring IgG Fc N-glycosylation changes during treatment. This would potentially provide information on the effectiveness of the treatment and offer the possibility to adjust the dose or treatment frequency for a specific patient. Within the pharmaceutical industry high-throughput glycopeptide profiling may prove its value during quality control and might be particularly useful for monitoring the fermentation process. This then allows early identification of harmful glycosylation changes and offers the possibility to interfere with the production process and restore product quality without losing an entire harvest.

As already mentioned the glycosylation of therapeutic proteins has to be human-like to prevent adverse effects. Therefore, prior to marketing complete structural characterization of protein glycosylation is mandatory for every biopharmaceutical. Here, a strategy based on MS can be problematic as no glycan linkage information is obtained and discrimination of isomers is virtually impossible even when cross ring fragment information is obtained.^{191,248} To obtain such structural and linkage information MS may be combined with lectin chromatography, and/or endo- and exoglycosidase treatment. High-throughput protein glycosylation analysis by MS with analysis techniques such as those developed in this thesis is, therefore, only useful with sufficient pre-knowledge on the glycosylation features of the specific protein. It should

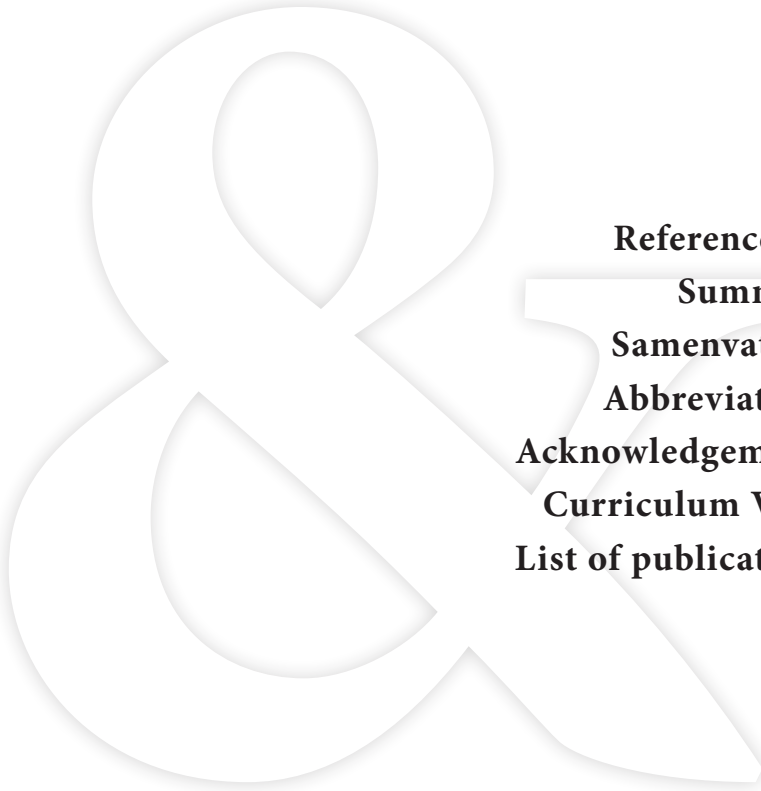
be kept in mind that hitherto no technique is available which is capable of elucidating the entire structural complexity of protein glycosylation in a single analysis, and for detailed structural analysis the information from several (complementary) strategies has to be combined.

To our knowledge, the Fc N-glycosylation sites of human polyclonal IgG are fully occupied. In cases where only partial occupation of N-glycosylation sites occurs (e.g. recombinant expressed proteins)¹⁸⁹ it might be required to monitor both glycosylated peptides and non-glycosylated variants of the peptides carrying the N-glycosylation consensus sequence. For these situations RP-SPE is the most suitable purification method as glycosylated and non-glycosylated variants of the peptides tend to co-elute. However, care should be taken with such an approach as MS glycopeptide signals are easily suppressed by co-eluting peptides which often possess higher proton affinities. Moreover, the acidic conditions applied during elution of (glyco)peptides from reversed phase stationary phases can quickly lead to partial desialylation of the glycopeptides. By contrast, HILIC-SPE is highly suited for glycoproteins with a high level of sialylation as elution from HILIC stationary phases can be performed with water. Moreover, HILIC purifications such as those described in **Chapter 3** and **Chapter 4** of this thesis allow very selective glycoconjugate enrichment and at the same time the removal of salts, most non-glycosylated peptides, and detergents from glycoconjugate samples.

In this thesis we predominantly performed targeted analyses where we selectively studied Fc N-glycosylation of purified IgG samples and did not gather other potentially useful information. With this strategy we effectively determined IgG Fc N-glycosylation changes occurring during pregnancy (**Chapter 2**), aging (**Chapter 4**), vaccination (**Chapter 6**) and two autoimmune diseases (**Chapter 7**). Our results of clinical cohorts confirmed the previously reported IgG glycosylation changes with age, sex and pregnancy,^{4,8-10,13,14} and revealed specific glycosylation changes with vaccination and the two autoimmune diseases (i.e. LEMS and MG). However, no conclusive information on the regulatory mechanisms involved in IgG Fc N-glycosylation changes or on the immunological role and implications of these alterations have been obtained. While effector immune mechanisms of IgG are modulated by the Fc N-glycosylation, binding of the antigen is influenced by glycosylation of the Fab portion as has already been shown for asymmetric antibodies in pregnancy.²¹² By developing high-throughput techniques capable of simultaneously analyzing IgG Fc and Fab glycosylation more conclusive information might be obtained on the immune-regulatory and functional aspects of IgG glycosylation. Moreover, by broadening the range of simultaneously monitored blood glycoproteins (e.g. circulating lysosomal hydrolases,²⁴⁹ erythropoietin, fibrinogen, apolipoproteins, tissue plasminogen activator, fetuin, transferrin etc.) additional information on disease related glycosylation changes may be revealed. For complex biological samples the use of LC-MS is preferred over MALDI as the complexity of the mass spectra is reduced by the LC separation, and the retention time provides an additional criterion for the identification of glycopeptides apart from their

m/z. The more sophisticated ultra high pressure LC separation systems coupled to high resolution mass spectrometers are expected to improve chromatographic resolution and confidence with glycopeptide identification, and will allow analysis of more complex glycopeptide samples.

With current technologies, targeted analysis and de novo glycan sequencing in complex biological samples remains a challenging and tedious task due to the lack of computational tools (e.g. algorithms, databases) capable of automated interpretation of high-throughput MS and MS/MS data from glycoconjugates. Hitherto, computational tools (e.g. GlycoPep DB,²⁵⁰ GlycoSpectrumScan,²⁵¹ GlycoPeptideSearch) are available which facilitate compositional assignment for glycopeptides by comparing experimentally measured masses to calculated glycopeptide masses from carbohydrate databases. However, MS/MS spectra often do not reveal the complete structure information of the glycan. Furthermore, the quality of glycopeptide fragmentation spectra strongly depends on the experimental settings and can substantially differ between different MS instruments. Therefore, intensive manual evaluation remains required for correct assignment. To overcome these limitations a large database is required that contains good quality (high resolution) glycopeptide fragmentation mass spectra (preferably obtained with various instruments) of different biological and clinical samples. Together with a standardization of mass spectrometric procedures and settings, this would offer the possibility to match experimentally obtained MS/MS spectra with spectra in the database and might allow the association of specific glycosylation changes with a particular biological state in a more automated high-throughput fashion.



Reference list

Summary

Samenvatting

Abbreviations

Acknowledgements

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Summary

Immunoglobulins (Igs) represent an important part of the human immune system and can adapt to many different antigenic challenges. Structural variety of Igs is achieved by the expression of hypervariable regions which create the antigen binding site and define the antigen specificity and affinity. Moreover, multiple classes (i.e. IgM, IgD, IgG, IgA, IgE) and subclasses (e.g. IgG1-4) of immunoglobulins are present in humans, differing in temporal expression patterns as well as in function. IgG represents the most abundant antibody class in the human circulation, being present at a concentration of approximately 10 mg/ml. IgG consists of two heavy chains and two light chains. Parts of the heavy chains, together with the light chains, form two fragment antigen binding (Fab) moieties, whilst the remainders of the two heavy chains form the fragment crystallizable (Fc) moiety. Next to the enormous variety in the sequence of the antigen-binding region of plasma IgG, human IgGs vary in the N-glycan structure at the highly conserved N-glycosylation site asparagine 297 in the C_H2 domain of each heavy polypeptide chain of the Fc part. This N-glycan is often core-fucosylated and varies in the number of antenna galactose residues resulting in the prominent glycoforms G0F (no galactose), G1F (1 galactose), and G2F (2 galactoses). A small part of the Fc N-glycans may carry a bisecting N-acetylglucosamine (GlcNAc). In addition, these glycans may contain a sialic acid residue attached to an antenna galactose. Fully galactosylated N-glycans are positioned between the Fc polypeptide chains, resulting in an open Fc conformation which is required for high affinity binding to Fcγ receptors. Small changes in the Fc glycosylation pattern can already have a profound influence on the interaction of the Fc portion with receptors modulating the anti- and pro-inflammatory properties of IgG. Over the past 30 years, many descriptive data regarding the biological and clinical role of IgG Fc N-glycosylation have been accumulated. The biological role of IgG Fc N-glycosylation and strategies to purify and analyze the Fc N-glycosylation are reviewed in **Chapter 1**. In addition, the importance of the glycosylation on therapeutic antibodies is described.

Mass spectrometry provides great opportunities for detailed structural characterization of protein glycosylation including protein identification, determination of site-specific glycosylation profiles, and structural characterization of glycans at the level of released glycans and glycopeptides. In **Chapter 2** the development of a fast reversed phase (RP) nanoLC-MS system for IgG Fc N-glycosylation analysis is described. A robust system was obtained by coupling the fast nanoLC separation to the mass spectrometer by a sheath-flow ESI sprayer. For each of the IgG subclasses the eight major glycoforms showed an interday analytical variation of below 5%. The method was successfully applied for fast and detailed IgG subclass-specific profiling of the Fc N-glycosylation for 26 women at several time points during pregnancy and after delivery, confirming the previous reported increase of IgG galactosylation during pregnancy. The technique additionally allowed sensitive and accurate determination of sialylated IgG glycopeptides, revealing increased levels of IgG sialylation during pregnancy.

Compared to LC-MS, MALDI-MS analysis times are much shorter. For sensitive and detailed glycosylation analysis by MALDI-MS analysis, however, suitable sample preparation of glycopeptides is crucial as signals are easily suppressed by coexisting salts and peptides. Hydrophilic interaction liquid chromatography (HILIC) has been found to be very suitable for selective glycopeptide purification. In **Chapter 3** the development of filter-free cotton wool HILIC solid-phase extraction (SPE) microtips for the purification of glycoconjugates is described. The reproducibility of pristine and reused tips was extensively evaluated, showing a low interday variability for the major IgG glycoforms. The cotton wool microtips showed a high capacity (at least 5 μ g 2-AA labeled maltoheptaose per 500 μ g cotton) and were successfully applied for the extraction and purification of glycopeptides and released glycans from complex samples.

In **Chapter 4** of this thesis the potential of 96-well plate format purification strategies for reproducible high-throughput sample preparation and detailed MALDI analysis of IgG Fc N-glycosylation in the human circulation is described. It is shown that 96-well plate format IgG purification with protein A affinity chromatography combined to RP or HILIC SPE purification of the tryptic glycopeptides provided very reproducible and detailed subclass-specific IgG Fc N-glycosylation profiles with a low interday variability (below 5% for the six major IgG1 and IgG2 glycoforms). The occurrence of massive in-source and metastable decay for sialylated glycopeptides during MALDI-TOF-MS limited high resolution analysis to neutral glycopeptides only. By contrast, MALDI-FTICR-MS equipped with an intermediate pressure MALDI source proved to be very suited for fast and robust high resolution profiling of intact IgG Fc N-glycopeptides including sialylated glycopeptides. The suitability of Sepharose HILIC SPE and MALDI-FTICR-MS for IgG subclass-specific high-throughput Fc N-glycosylation profiling from human plasma was successfully shown for a cohort of 62 human samples, confirming the previously described age- and sex-related IgG glycosylation changes.

The choice of the MALDI matrix is crucial for minimizing desialylation of glycoconjugates by mass spectrometric in-source and metastable decay. For example, CHCA tends to lead to an almost complete loss of sialic acids, while the “colder” character of DHB often limits sialic acids loss, particularly in combination with negative-ion mode and/or linear-mode MALDI-TOF-MS. In MALDI sample preparation, DHB however tends to form large needle-like crystals. This macrocrystalline nature generally results in areas where certain analytes are highly concentrated, and in a very large spatial heterogeneity of mass spectra obtained from one sample spot. Moreover, MALDI-TOF-MS of macrocrystalline sample preparations is known to result in mass spectra of rather low resolution and mass accuracy in linear oriented MALDI-TOF instruments. In **Chapter 5** the potential of the microcrystalline 4-chloro- α -cyanocinnamic acid (Cl-CCA) matrix for MALDI-TOF-MS detection of labile sialylated glycoconjugates has been evaluated. The microcrystalline nature of the Cl-CCA matrix with homogeneous spatial distribution resulted in a high shot-to-shot reproducibility with good resolution

and mass accuracy. Both DHB and Cl-CCA allowed sensitive and reproducible negative ion mode MALDI-TOF-MS analysis of sialylated glycopeptides. The Cl-CCA matrix proved to be as effective as DHB in preventing sialic acid loss during MALDI-TOF-MS, which was illustrated by the analysis of sialylated N- and O-glycoconjugates.

Antibody effector functions are modulated by the attached Fc N-glycans. In response to specific stimuli, different glycosylation profiles can be obtained at the level of antigen-specific IgG in comparison to glycosylation at the level of total IgG. In **Chapter 6** IgG Fc N-glycosylation changes induced by vaccination of 10 Caucasian adults and 10 African children were studied. While vaccination was found to have no effect on the glycosylation of total IgG, anti-vaccine IgG1 showed increased levels of galactosylation and sialylation and a decreased level of bisecting N-acetylglucosamine upon active immunization. The data indicate that the infectious agent will encounter IgGs with quite different glycosylation profiles depending on the vaccination time point which might influence the antibody effector functions relevant to immunity. However, it should be stressed that the regulatory aspects and functional implications of human IgG glycosylation features are still largely unknown, and that further research is needed.

Shifts in IgG Fc N-glycosylation features in well-defined antibody-mediated autoimmune diseases could provide valuable insights into which IgG Fc N-glycosylation features potentially influence antibody pathogenicity. In **Chapter 7** IgG Fc N-glycosylation changes have been evaluated for the neurological disorders Lambert-Eaton myasthenic syndrome (LEMS), and myasthenia gravis (MG). LEMS and MG patients showed lower levels of IgG2 galactosylation compared to controls. In addition, the level of IgG1 galactosylation in LEMS patients was lower compared to the controls. Interestingly, LEMS patients below 50 years showed elevated levels of bisecting N-acetylglucosamine on IgG1 and IgG2, demonstrating for the first time the link of changes in the level of bisecting N-acetylglucosamine with disease. However, the immunological relevance of the observed IgG glycosylation changes remains unclear and future longitudinal studies of total and autoantigen-specific IgG Fc N-glycosylation are required to reveal possible associations between disease severity and glycosylation features.

In this thesis novel approaches for fast, miniaturized and high-throughput analysis of IgG Fc N-glycosylation are presented, and the utility of these methods has been demonstrated for clinically relevant research questions. The limitations, strengths and perspectives of these techniques are discussed in **Chapter 8**.

Samenvatting

Immunoglobulinen (Igs) vertegenwoordigen een belangrijk deel van het menselijk immuunsysteem en worden geproduceerd als reactie op lichaamsvreemde stoffen (antigenen) aanwezig op pathogenen zoals virussen en bacteriën. Doordat de Igs deze antigenen herkennen, kunnen pathogenen onschadelijk worden gemaakt. In het menselijk lichaam komen meerdere Ig klassen (i.e. IgM, IgD, IgG, IgA, IgE) en subklassen (e.g. IgG1-4) voor, waarvan IgG de hoogste concentratie heeft in de circulatie (ca. 10 mg/ml bloed). IgG bestaat uit twee identieke lichte en twee identieke zware aminozuurketens die elk constante delen en een variabel deel bevatten. De lichte aminozuurketens vormen samen met het variabele deel en een constant deel van de zware aminozuurketens twee antigeen bindende (Fab) delen. De resterende constante delen van de zware aminozuurketens (C_{H2} en C_{H3}) vormen het fragment crystallizable (Fc) gedeelte wat verantwoordelijk is voor de binding met de Fc γ receptoren op effector cellen. In het Fc gedeelte zit op de sterk geconserveerde N-glycosylerings site asparagine 297 in het C_{H2} domein van beide zware aminozuurketens een suikerketen (N-glycaan). N-glycanen hebben een gemeenschappelijk bi-antennaire kern (chitobiose kern) en variëren in het aantal antenne galactoses. Een groot gedeelte van de N-glycanen heeft een fucose aan het met het asparagine residu verbonden N-acetylglucosamine. Een klein percentage van de N-glycanen heeft een siaalzuur aan de antenne galactose(s) en/of een additionele N-acetylglucosamine (bisecting N-acetylglucosamine). Doordat de suikerketens van volledig gegalactosyleerde N-glycanen naar elkaar toe wijzen ontstaat een open Fc structuur die noodzakelijk is voor een sterke binding met de Fc γ receptoren. Kleine veranderingen in de samenstelling van de Fc N-glycanen kunnen een grote invloed hebben op de binding met de Fc γ receptoren en kunnen hierdoor de anti- en pro-inflammatoire eigenschappen van IgG veranderen. In de laatste 30 jaar is veel onderzoek verricht naar de precieze biologische rol van IgG Fc N-glycosylering. In **hoofdstuk 1** zijn de belangrijkste bevindingen en analyse technieken van de voornaamste studies samengevat. Tevens wordt het belang van de glycosylering van therapeutische antilichamen beschreven.

Een van de meest potentiële technieken voor de gedetailleerde structurele karakterisering van IgG Fc N-glycosylering is vloeistof chromatografie (LC) gekoppeld aan massaspectrometrie (MS). In **hoofdstuk 2** van dit proefschrift is de ontwikkeling van een snelle reversed phase (RP) nanoLC-MS methode voor de analyse van IgG Fc N-glycosylering beschreven. Door de nanoLC te koppelen aan de MS met een “sheath-flow” electrospray (ESI) sprayer is een robuust en gevoelig systeem verkregen. Voor de acht meest voorkomende Fc N-glycopeptiden van IgG1 en IgG2 was de inter-dag analytische variatie kleiner dan 5%. De methode is succesvol toegepast voor de analyse van een cohort van 26 zwangere vrouwen en de verkregen resultaten bevestigen de in de literatuur gerapporteerde verhoging in IgG galactosylering tijdens de zwangerschap. Daarnaast werd door de gevoelige meting van gesialyleerde glycopeptiden een verhoging in de sialylering van IgG waargenomen tijdens de zwangerschap.

In vergelijking met LC-MS zijn de analyse tijden met matrix-assisted laser desorptie ionisatie (MALDI) MS vele malen korter. Glycopeptide signalen kunnen echter gemakkelijk worden onderdrukt door andere moleculen in het mengsel zoals zouten en peptiden zonder glycanen. Voor een gevoelige en gedetailleerde MALDI-MS analyse is de monstervoorbereiding dan ook cruciaal. Door de hydrofiele eigenschappen van de N-glycanen kunnen glycopeptiden zeer specifiek worden gezuiverd met hydrofiele interactie vloeistof chromatografie (HILIC). In **hoofdstuk 3** van dit proefschrift is de ontwikkeling van watten HILIC microtips voor de vaste fase extractie (SPE) van glycoconjugaten beschreven. De reproduceerbaarheid van nieuwe en hergebruikte microtips laat voor de meest voorkomende IgG Fc N-glycopeptiden een lage inter-dag analytische variatie zien. De watten microtips hebben een hoge capaciteit (minimaal 5 µg 2-AA labeled maltoheptaose per 500 µg wat) en konden succesvol worden toegepast voor de extractie en zuivering van glycopeptiden en afgesplitste glycanen uit complexe mengsels.

In **hoofdstuk 4** van dit proefschrift is de geschiktheid van RP (i.e. C18) en HILIC (i.e. Sepharose, cellulose) SPE in 96-well plaat format beschreven voor de high-throughput zuivering van IgG Fc N-glycopeptiden. Voor de zes meest voorkomende Fc N-glycopeptiden van IgG1 en IgG2 was de inter-dag analytische variatie van de monstervoorbereidingen kleiner dan 5%. De monsters zijn met zowel MALDI-TOF-MS als intermediate pressure MALDI-FTICR-MS gemeten. Tijdens MALDI-TOF-MS analyse vindt op grote schaal in-bron en metastabiel verval van gesialyleerde glycopeptiden plaats, waardoor het niet mogelijk is om deze met hoge resolutie te meten. De MALDI-FTICR-MS bleek echter uitermate geschikt voor de hoge resolutie analyse van de gesialyleerde en neutrale glycopeptiden. De Sepharose HILIC-SPE met MALDI-FTICR-MS detectie is succesvol toegepast voor de analyse van IgG Fc N-glycopeptiden van 62 humane controle monsters. De verkregen resultaten bevestigen de leeftijd en geslacht gerelateerde IgG Fc N-glycosylerings veranderingen zoals beschreven in de literatuur.

De keuze van de MALDI matrix is cruciaal voor het minimaliseren van in-bron en metastabiel verval van gesialyleerde glycopeptiden. α-Cyano-4-hydroxycinnamic zuur (CHCA) als hete matrix leidt bijvoorbeeld tot een vrijwel volledig verlies van de siaalzuren. Dit kan worden beperkt door gebruikt te maken van een koude matrix zoals 2,5-dihydroxybenzoëzuur (DHB). Tijdens de standaard MALDI monstervoorbereiding heeft DHB echter de neiging om grote naaldachtige kristallen te vormen. Deze eigenschap resulteert vaak in sweet spots (lokale plaatsen in het kristal die rijk aan monster zijn) en in een relatief lage resolutie en massa nauwkeurigheid met lineair georiënteerde MALDI-TOF-MS instrumenten. In **hoofdstuk 5** is de potentie van de microkristallijne matrix 3-(4-chloor-fenyl)-2-cyano-acrylzuur (Cl-CCA) geëvalueerd voor de MALDI-TOF-MS analyse van gesialyleerde glycoconjugaten. De zeer homogene ruimtelijke verdeling van de Cl-CCA microkristallen resulteerde in een hoge shot-to-shot reproduceerbaarheid met een goede resolutie en massa nauwkeurigheid voor de

IgG glycopeptiden. Met zowel DHB als Cl-CCA was een gevoelige en reproduceerbare negatieve ion mode MALDI-TOF-MS analyse van gesialyleerde glycopeptiden mogelijk. De Cl-CCA matrix bleek even efficiënt voor het minimaliseren van siaalzuur verlies als de DHB matrix, wat is geïllustreerd aan de hand van de analyse van N- en O-glycoconjugaten.

IgG effector functies worden beïnvloed door de Fc N-glycosylering. Tijdens stimuli kunnen de IgG Fc N-glycoprofielen op totaal IgG niveau verschillen ten opzichte van de IgG Fc N-glycoprofielen op antigeen-specifiek niveau. In **hoofdstuk 6** zijn de veranderingen in Fc N-glycosylering van totaal IgG en antigeen-specifiek IgG onderzocht voor en na vaccinatie van 10 Kaukasische volwassenen en 10 Afrikaanse kinderen.

Terwijl er bij vaccinatie geen effect zichtbaar was voor de Fc N-glycosylering op totaal IgG niveau, was bij anti-vaccin specifiek IgG1 de galactosylering en sialylering verhoogd en de hoeveelheid bisecting N-acetylglucosamine verlaagd. De resultaten tonen aan dat afhankelijk van het vaccinatie tijdstip een ziekteverwekker IgGs zal tegenkomen met zeer verschillende Fc N-glycoprofielen die een rol kunnen spelen bij antilichaam effector functies en immuniteit. Verder onderzoek is noodzakelijk om de regulerende aspecten en functionele implicaties van IgG Fc N-glycosylering op te helderen.

Verschuivingen van IgG Fc N-glycosylerings profielen in goed gedefinieerde auto-immuunziekten waarbij antilichamen een belangrijke rol spelen kunnen waardevolle inzichten bieden in welke glycosylerings kenmerken de antilichaam pathogeniciteit kunnen beïnvloeden. In **hoofdstuk 7** zijn IgG Fc N-glycosylerings veranderingen bestudeerd bij de neurologische aandoeningen Lambert-Eaton myasthenic syndroom (LEMS) en myasthenia gravis (MG). Ten opzichte van de onaangedane controles was de IgG2 galactosylering in LEMS en MG patiënten lager. Bij LEMS patiënten werd tevens een lagere IgG1 galactosylering waargenomen dan die bij de controlegroep en was het niveau van de IgG1 en IgG2 bisecting N-acetylglucosamine voor patiënten onder de 50 jaar hoger. De immunologische relevantie van de waargenomen IgG glycosylerings veranderingen is echter nog onduidelijk. Om eventuele associaties van Fc N-glycosylering met de ernst van de ziekte op te helderen is longitudinaal onderzoek waarbij gekeken wordt naar totaal en autoantigeen-specifieke IgG noodzakelijk.

In dit proefschrift worden nieuwe methoden gepresenteerd voor snelle, geminiaturiseerde en high-throughput analyse van IgG Fc N-glycosylering. Verder wordt het nut van deze methoden aangetoond aan de hand van klinisch relevante onderzoeksvragen. De beperkingen, sterke kanten en perspectieven van deze methoden worden besproken in **hoofdstuk 8**.

List of abbreviations

2-AA	2-aminobenzoic acid
3-AA	3-aminobenzoic acid
2-AB	2-aminobenzamide
AChR	postsynaptic muscle nicotinic acetylcholine receptors
ACN	acetonitrile
ADCC	antibody-dependent cellular cytotoxicity
ANOVA	analysis of variance
APTS	8-aminopyrene-1, 3, 6-trisulphonate
BSA	bovine serum albumin
CDC	complement-dependent cytotoxicity
CHCA	α -cyano-4-hydroxycinnamic acid
CID	collision induced dissociation
CIEX	cation exchange
Cl-CCA	3-(4-chloro-phenyl)-2-cyano-acrylic acid), 4-chloro- α -cyanocinnamic acid
DC-SIGN	Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin
DHB	dihydroxybenzoic acid
DSS	disease severity scores
DTT	dithiothreitol
ESI	electrospray ionization
FA	formic acid
Fab	fragment antigen binding
Fc	fragment cristallizable
Flu	fluorescence detection
FTICR	Fourier transform ion cyclotron resonance
Fuc	fucose
Gal	galactose
GlcNAc	N-acetyl glucosamine
Glu-C	endoproteinase Glu-C and V8 protease
G _n F	biantennary N-glycan with n galactoses and a core fucose
G _n F S _n	biantennary N-glycan with n galactoses, a core fucose and n sialic acids
G _n F NS _n	biantennary N-glycan with n galactoses, a core fucose, an N-acetyl glucosamine and n sialic acids
Hex	hexose
HexNAc	N-acetylhexosamine
HILIC	hydrophilic interaction liquid chromatography
IgG	immunoglobulin G
IPA	isopropanol.

IVIg	intravenous immunoglobulin
LEMS	Lambert-Eaton myasthenic syndrome
LIF	laser-induced fluorescence
LOD	limit of detection
Lys-C	lysylendopeptidase
<i>m/z</i>	mass over charge ratio
mAb	monoclonal antibody
MALDI	matrix assisted laser desorption
MG	myasthenia gravis
MS	mass spectrometry
MuSK	muscle-specific kinase
NANA	N-acetyl neuraminic acid
NeuGc	N-glycolylneuraminic acid
NP-40	nonidet P-40
OVA	ovalbumin
PA	propionic acid
PBS	phosphate buffered saline
PGC	porous graphitic carbon
PMP	1-phenyl-3-methyl-5-pyrazolone
PNase F	N-glycosidase F
QIT	quadrupole ion trap
RA	rheumatoid arthritis
RCA-I	ricinus communis agglutinin-I
RP	reversed phase
RSD	relative standard deviation
RT	room temperature
S/N	signal to noise ratio
SA	sialic acid
SDS	sodium dodecyl sulfate
SNA	Sambucus nigra agglutinin
SPE	solid phase extraction
STD	standard deviation
TAT	total analysis time
TFA	trifluoroacetic acid
TOF	time of flight
TPCK	tosyl phenylalanyl chloromethyl ketone
VGCC	voltage-gated calcium channels
ZIC-HILIC	zwitterionic-type hydrophilic interaction liquid chromatography

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Curriculum Vitae

Maurice Henricus Johannes Selman was born at the 12th of April 1980 in Heteren the Netherlands. Already as a child chemistry had something exciting and spoke to his imagination. This imagination never completely left and made that he started studying Toxicology at the Hogeschool Larenstein in Velp. In February 2003 he finished the Bachelor of Science and started working as a technician for 6 months at the Veterinary Pharmacy Pharmacology and Toxicology department of the Utrecht University where he performed research to mycotoxins in feed and strategies to prevent their detrimental effects in livestock. In August 2003 he continued his study at the Vrije Universiteit in Amsterdam, where he studied Pharmaceutical Sciences with a major in Toxicology and a minor in Bioanalytical Chemistry. After graduation he started working as a junior researcher at the Institute of Biodiversity and Ecosystem Dynamics of the University of Amsterdam where he performed research to stable UV-B absorbing components in the sporopollenin fraction of pollen. In May 2006 he started as a technician at the department of Biomedical Analysis of the Utrecht University where he developed separation techniques for metabolomics purposes. In January 2008 he got the opportunity to start his PhD entitled: "Glycosylation analysis of immunoglobulin G: method development and clinical applications" in collaboration with Roche Diagnostics, at the Glycoproteomics section, Department of Parasitology of the Leiden University Medical Center. In January 2012 he will continue the collaboration of the Glycoproteomics section and Roche as a post-doc.

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