High-throughput mass spectrometric N-glycomics

Reiding, K.R.

Part I – Introduction

Chapter 1

General introduction

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The human body is an intricate and dazzling piece of machinery, with interlocking systems in place to allow us to do the things we want to do in daily life. In all of these systems we have the presence of countless large and small molecules, with only their combined action leading ultimately to a macrobiological phenotype such as fighting a disease, digesting lunch, or stretching your arm. Workhorses involved in this machinery are commonly proteins, which can cover a wide array of functions which depend on their precise makeup, from transport *via* replication to catalysis. The function, localization, and control of proteins is greatly enhanced by modifications they may undergo, prominent examples being phosphorylation and acetylation, but in the extracellular domain none of these is as abundant as the decoration of proteins with carbohydrate structures, coined glycosylation[1,2].

1.1 Glycosylation

Glycosylation, the reaction of a carbohydrate with another molecule, is an important way in which biomolecules can be modified. Enzymatic glycosylation may occur on lipids, proteins and on other carbohydrates, and respectively leads to the formation of glycolipids, glycoproteins and glycans (although "glycan" may also refer to the carbohydrate content of a glycoconjugate)[3]. Glycosylation is a wide-spread process in biology, and the presence or absence of a glycan (macroheterogeneity) or the actual size and structure thereof (microheterogeneity) are known to have major effects on a glycoconjugate. Glycans have for example been shown to affect glycoprotein folding and quality control[4-6], solubility[7], spatial organization[8], and receptor interaction[9,10], but many more functions have been described[11].

Human protein glycosylation occurs in two main forms, namely *N*-glycosylation and *O*-glycosylation, which differ by the type of linkage between the carbohydrate and the protein backbone, as well as by the amino acids on which they can occur[3,12]. *N*-Glycosylation, which yields *N*-glycans, is the enzymatic attachment of an oligosaccharide by *N*-glycosidic linkage to the nitrogen of the side chain amide of an asparagine. This process predominantly occurs within the amino acid consensus sequence Asp-X-Ser/Thr, where X can be any amino acid except proline[3,13]. On the other hand, *O*-glycosylation, yielding *O*-glycans, is the attachment of a monosaccharide by *O*-glycosidic linkage to the oxygen of the side-chain hydroxyl group, which may occur on serine or threonine in case of mucin-type *O*-glycosylation (although modification of hydroxylysine and tyrosine have also been described)[3,14,15]. After initial attachment, both *N*- and *O*-glycans can be further modified by glycosyltransferases and glycosidases, *i.e.*, enzymes that respectively add and remove monosaccharides, eventually resulting in a wildly divergent set of glycoforms that can be achieved for a given protein[3].

Both *N*- and *O*-glycosylation can be regarded as post-translational protein modification (PTM), sharing the nomenclature with, *e.g.*, phosphorylation and acetylation[2]. *N*-Glycosylation is sometimes also referred to as co-translational, for the reason that initial *N*-glycan attachment takes place during (and not after) translation of a protein into the rough endoplasmic reticulum (ER) lumen[3,12]. Among the known PTMs found in the extracellular space, *N*-glycosylation is estimated to be the most abundant, and the majority of plasma proteins are thought to be glycosylated with at least one *N*-glycan[1,2]. Nevertheless, it remains one of the most heavily under-studied biological topics to date, owing to the structural complexity involved and the resulting analytical challenges.

1.2 Monosaccharide structure

Glycans are polymers made up of monosaccharides just like deoxyribonucleic acid (DNA) and proteins are respective biopolymers of nucleotides and amino acids. However, whereas DNA and peptides are typically linear sequences, glycans tend to be arranged in complex branching structures, and in addition often show many kinds of stereoisomerism[3].

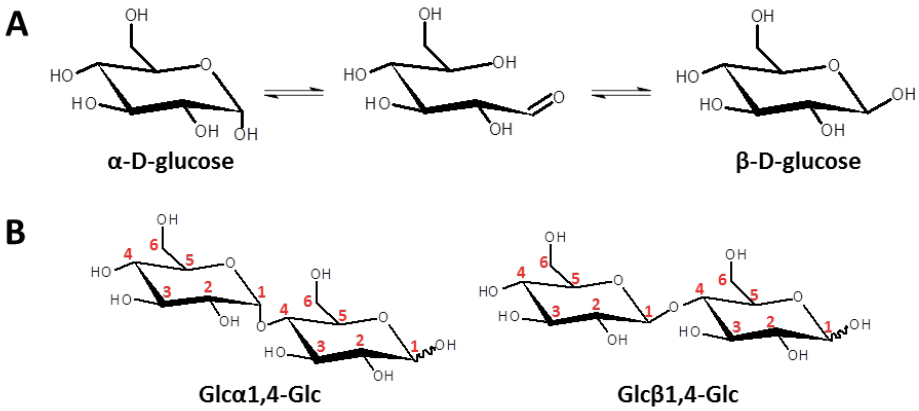

***Figure 1**. **A)** The α- and β-anomers of cyclic D-glucose and their open intermediate. **B)** Two glucose residues in either α1,4-linkage or β1,4-linkage, with numbered carbons. A perturbed line has been used to indicate the anomeric hydroxyl group involved in ring opening and closing.*

Taking as example the aldohexose, a group of monosaccharide isomers with six carbons and an aldehyde at the first carbon (chemical formula $C_6H_{12}O_6$), the presence of four chiral centers means that already 16 (2^4) stereoisomers are possible. The stereoisomerism at the fifth carbon is denoted by the letter substitution D or L, *e.g.*, D-aldohexose, while the stereoisomerism at the three remaining chiral carbons defines the overall name of the molecule, *e.g.*, glucose, mannose or galactose[3]. Free monosaccharides alternate in solution between an open-chain form and a cyclic form, but it is the cyclic form that is predominantly found in polysaccharides (**Figure 1A**)[3]. Cyclization of an aldohexose may either occur with the hydroxyl group at the fourth carbon, forming a furanose (*e.g.*, glucofuranose), or with

the hydroxyl group at the fifth carbon, forming a pyranose (*e.g.*, glucopyranose)[3]. In both cases this leaves the first carbon a new chiral center, of which the two configurations, named anomers, can be indicated with an α or β prefix (*e.g.*, β-glucopyranose)[3].

Polysaccharides, or glycans, are formed when monosaccharides are covalently joined by an *O*-glycosidic bond. While this could result from a condensation reaction between the anomeric carbon of a given monosaccharide with the hydroxyl group of another (such as the case with glycation), for glycosylation the bond is formed by the enzymatic transfer of a monosaccharide from a nucleotide-activated sugar. For example, beta-1,4-galactosyltransferase 1 may catalyze the transfer of the galactose of uridine diphosphate galactose (UDP-galactose) to glucose to form lactose (milk sugar)[16]. A resulting *O*-glycosidic linkage is typically described by the anomerism and carbons involved in the linkage, *e.g.*, α1,2 for a linkage between the first carbon of α-D-glucopyranose and the second carbon of D-fructofuranose to form sucrose (table sugar) (**Figure 1B**)[3]. An example underlining the functional differences between glycosidic linkage types can be found in glucose polymers, which when α1,4-linked forms amylose (a main component of starch), or when β1,4-linked instead forms cellulose (a main component of cotton and wood)[3].

Next to displaying stereoisomerism, monosaccharides in biology are often substituted at various hydroxyl groups with a wide range of other functional group. Examples of this include amination, amine acetylation, or the presence of a carboxylic acid group, which would for glucose respectively form glucosamine, *N*-acetylglucosamine, and glucuronic acid[3].

In all, given the total variety of monosaccharides available for polymerization, the number of potentially different glycan structures is truly staggering[17]. In reality, however, the types of monosaccharides involved in human protein glycosylation are rather constrained, and appear to be under tight control. The most common monosaccharides found in human *N*- and *O*-glycosylation are pyranoses, and principally comprise the deoxyaldohexose (dHex) L-fucose (Fuc), the aldohexoses (Hex) D-glucose (Glc), D-mannose (Man) and D-galactose (Gal), the *N*-acetylaldohexosamines (HexNAc) *N*-acetyl-D-glucosamine (GlcNAc) and *N*-acetyl-D-galactosamine (GalNAc), and the heavily substituted ketononose *N*-acetylneuraminic acid (Neu5Ac, belonging to the family of sialic acids) (**Figure 2**)[3,18]. The polymerization of these monosaccharide residues are what make up most of the human *N*- and *O*-glycans.

Whereas *N*- and *O*-glycosylation can be found within nearly all forms of life, different monosaccharides can be found within non-human glycan structures. For example, aldopentoses like D-xylose are commonly seen on the *N*-glycans of plants and parasites[3,20], and the mouse largely incorporates the sialic acid *N*-glycolylneuraminic acid (Neu5Gc) instead of *N*-acetylneuraminic acid[3,18].

1.3 The biosynthesis of protein *N*-glycosylation

The work presented in this thesis mainly involves *N*-glycosylation which, as prior indicated, can be regarded as both a post-translational and co-translational modification[3,12]. During protein synthesis into the lumen of the rough ER, *N*-glycosylation occurs when the oligosaccharide portion of a membrane-bound glycolipid, dolichol-$Glc_3Man_9GlcNAc_2$ (Glcα1,2-Glcα1,3-Glcα1,3-Manα1,2-Manα1,2-Manα1,3-[Manα1,2-Manα1,3-[Manα1,2-Manα1,6-]Manα1,6-]Manβ1,4-GlcNAcβ1,4-GlcNAcβ), is transferred in its entirety to the asparagine of a polypeptide containing the appropriate consensus sequence (Asn-X-Ser/Thr)[3,12]. The glucose residues of the then *N*-linked glycan are subsequently trimmed down by the action of glucosidase I and II, but the last glucose can repeatedly be removed and added (by glucosyltransferase activity) while the glycoprotein undergoes folding[3,12]. The presence of a monoglucosylated *N*-glycan facilitates interaction with the proteins calnexin and calreticulin, which form an important part of the protein quality control mechanism of the ER[3,12]. This glycan-mediated mechanism serves as checkpoint to decide between clearing proteins for further processing (requiring the persistent removal of the glucose) or alternatively sending them to the lysosomal pathway of protein degradation[12,21].

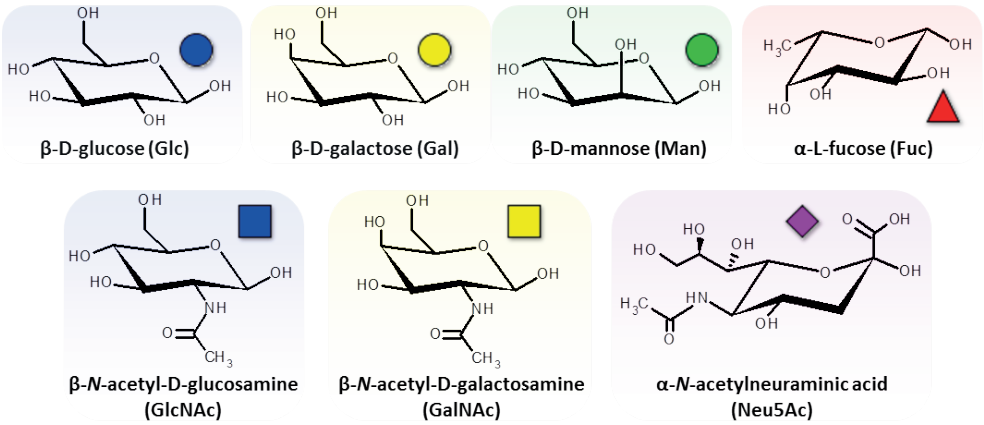

***Figure 2**. **A)** Monosaccharides commonly involved in human N- and O-glycosylation and their symbolical nomenclature as defined by the Consortium for Functional Glycomics (CFG)[19].*

From this point on, the type of *N*-glycosylation ($Man_9GlcNAc_2$) found on proteins may be conserved, eventually appearing as such in the extracellular space, or the glycan can be further processed in a variety of ways as the conjugate is guided through the ER and Golgi apparatus[3,12]. The processing of the glycan is not template-defined, and will ultimately depend on a multitude of factors, including the abundance of glycosidases and glycosyltransferases, the accessibility of the glycan for enzymatic alteration, the presence

of nucleotide sugars to serve as monosaccharide donor, the time spent in different compartments of the ER and Golgi, and so forth[3,12].

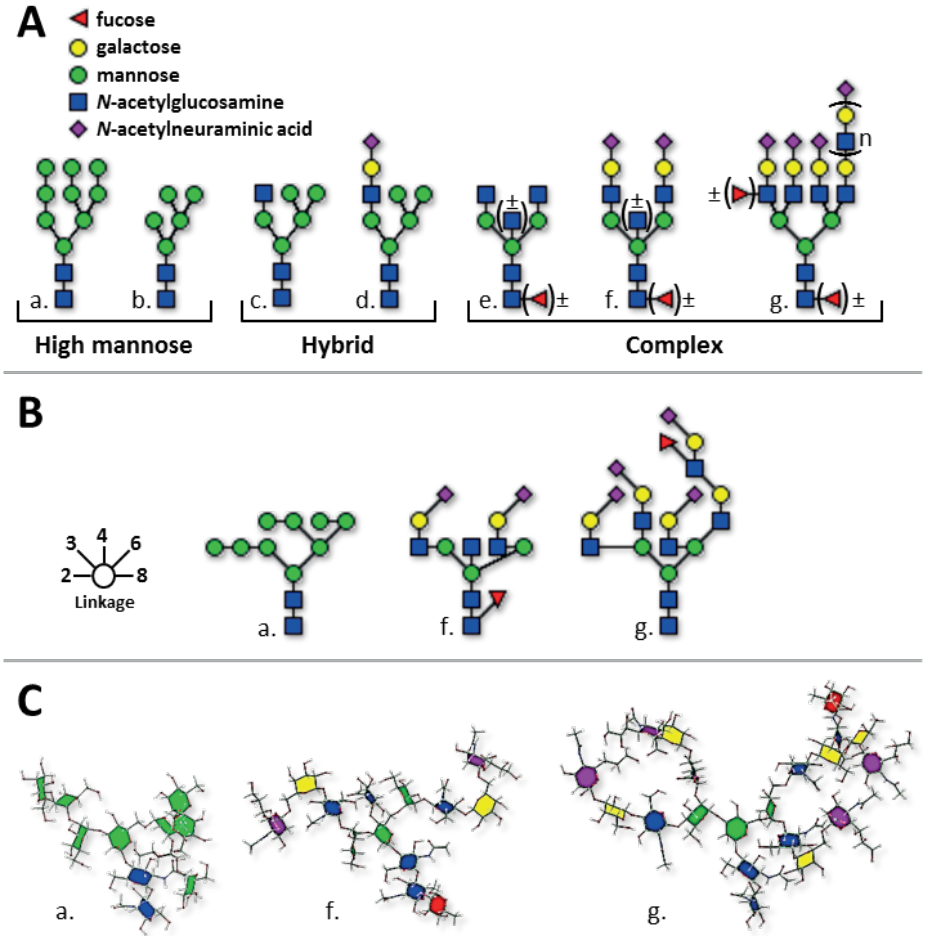

***Figure 3**. **A)** Typical N-glycan species as found on human plasma proteins. Distinction is made between high-mannose-type species (a. and b.), hybrid-type species (c. and d.) and complex-type species (e., f. and g.). This visualization style is simplified with regard to structure, and is commonly used to denote glycan compositions of which limited structural information is available. **B)** Species a., f. and g. with linkage information indicated by the angle. **C)** N-glycan structures for species a., f. and g, as edited from the Glycam structure prediction tool (glycam.org). Displayed here are the structures belonging to the lowest energy state, but glycans are highly mobile and will not maintain a static structure[3].*

In case of further processing, the glycan may be truncated to a $Man_5GlcNAc_2$ structure[3,12], after which an "antennary" GlcNAc residue can be appended in β1,2-linkage to the α1,3-linked mannose "branch" (referring to the branching mannoses on the β1,4-linked mannose) (**Figure 3**)[3,12]. The two mannoses remaining on the α1,6-linked branch may also be removed, and can be replaced by a β1,2-linked antennary GlcNAc[3,12]. An *N*-glycan is described as "high-mannose type" when both branches are occupied by mannose residues,

"hybrid type" when one of the branches has an antennary GlcNAc and the other contains mannoses, and "complex type" when both branches carry an antennary GlcNAc (**Figure 3**)[3,12]. Aside from having β1,2-linked antennae, human *N*-glycans may also have a β1,4-linked antenna on the α1,3-branch, and a β1,6-linked antenna on the α1,6-branch. These can form alternatively linked diantennary structures, or combine to yield tri- and tetraantennary species[3,12].

Antennary GlcNAcs can be extended in a variety of ways, the most common being the addition of a β1,4-linked galactose and subsequent addition of an *N*-acetylneuraminic acid (sialic acid) in either α2,6- or α2,3-linkage to the galactose[3,12]. Extension of structures may furthermore occur by α1,6-linked fucosylation of the innermost GlcNAc, by α1,2-, α1,3 or α1,4-fucosylation of antennae, by a "bisecting" GlcNAc that is β1,4-linked to the β1,4-linked core-mannose, by elongation of the β1,6-linked antenna to contain "LacNAc" repeats (Galβ1,4-GlcNAcβ1,3-), as well as by a variety of other modifications(**Figure 3**)[3,12]. Together this facilitates the highly diverse range of structures an *N*-glycan may have, potentially leading to specific oligosaccharide epitopes like sialyl-Lewis X (sLeX; Neu5Acα2,3-Galβ1,4-[Fucα1,3-]GlcNAc), a major ligand for E-selectins[3,22].

1.4 Glycosylation in health and disease

The process of glycosylation is not template-defined (such as DNA), but a considerable portion of the human genome is directly and indirectly involved in the process[23,24]. Genetic defects can lead to the absence of glycosylation on protein sequences where this is normally the case, a phenomenon named type I congenital disorder of glycosylation (CDG)[25]. Homozygous or compound heterozygous knock-outs are embryonically lethal, and individuals may otherwise suffer from severe symptoms including mental retardation and organ failure[25]. Genetic abnormalities leading to qualitative differences in glycosylation (*e.g.*, a decreased ability to form sialylated or fucosylated glycans) are grouped as type II CDG and may lead to a wide range of symptoms as well, including mental retardation, jaundice, and hypotonia[25]. Interestingly, some types of type II CGD appear treatable by dietary mannose, galactose or fucose supplementation, particularly when the genetic defect is in a nucleotide sugar pathway[25-28]. In both type I and type II CDGs, analysis of the glycan site occupancy and structures, either on specific proteins or within mixture of proteins such as in plasma, is highly useful for the detection and characterization of the defect.

Glycosylation may also be reflective of, or play a role in, tumor growth and metastasis. As glycosylation is ultimately produced by cells, aberrant regulation thereof, such as the case in cancer, may result in uncommonly glycosylated proteins being present in the circulation[29]. For example, while prostate cancer is diagnosed by the abundance of prostate-specific antigen (PSA) in the blood, it has also been shown that the type of glycosylation

present on the protein in circulation differs from that in the prostate, particularly by the degree of sialylation[30]. On the functional side, it is thought that the presence of glycan epitopes such as sLeX may be instrumental in allowing circulatory tumor cells to adhere to new positions, thereby facilitating tumor metastasis[29,31].

A setting in which glycosylation has shown diagnostic potential is in the discrimination of different types of diabetes. A particular genetic variant of the disease, *i.e.*, type 3 maturity-onset diabetes of the young (MODY 3), can often be distinguished from the other forms, *e.g.*, type I and II diabetes, by the decreased antennary fucosylation found on the *N*-glycosylation of plasma proteins[32]. This appears to be mainly driven by the *HNF1A* gene that is a risk factor for MODY, which is proposed to be a transcription factor for multiple genes responsible for antennary fucosylation (*e.g.*, *FUT3* and *FUT6*)[33]. The quantification of fucosylation is thought to be a simple and clinically interesting method for diabetes categorization, and may assist in the development of personalized treatment.

Glycosylation is also thought to play a large role in immunity and autoimmunity. Immunoglobulin G (IgG), the most abundant antibody in human plasma, serves as a major component in the adaptive immune system[34]. The protein generally carries one *N*-glycan on each of its two crystallizable fragment (Fc) domains, and displays *N*-glycosylation of the antibody-binding fragment (Fab) domains for around 20% of the circulatory IgGs[34,35]. The *N*-glycosylation of IgG-Fc has shown to heavily influence its Fc-gamma-receptor (FcγR) affinity, thereby modulating antibody-dependent cellular cytotoxicity (ADCC), with lack of a core-fucose leading to an up to 50-fold increased binding to FcγRIIIa and the degree of galactosylation and bisection having several-fold effect sizes[36-38]. Furthermore, various IgG-Fc glycosylation traits, notably galactosylation, have shown to associate with inflammation and autoimmune diseases such as rheumatoid arthritis (RA), in which lower galactosylation generally associates with higher degrees of inflammation[39-41]. Fab glycosylation is suspected to play a role in RA as well, as seen by the elevated degree of Fab-glycosylation present on disease-specific anti-citrullinated protein antibodies (ACPA) found in the subset of ACPA-positive RA patients[42]. The influence of glycosylation on antibody function extends to biopharmaceutical antibodies, meaning that production processes need to be under careful control and need to be monitored for glycan heterogeneity[43,44].

In addition to disease-associated glycosylation phenotypes, studies in healthy individuals have also shown numerous associations between glycosylation and phenotypes like age, sex, inflammation, body mass index, pregnancy and lipid levels, and so forth[45-48]. Interestingly, while the glycosylation within an individual is relatively stable, with changes usually being representative of a change in homeostasis, the variance between individuals is usually much greater[49,50]. This suggests a benefit for longitudinal study designs and implies that cross-sectional designs need to include considerable sample numbers.

1.5 Glycan analysis

While glycosylation is of clinical and biopharmaceutical importance, the analysis thereof is not easily performed. Since glycosylation does not have an equivalent to the polymerase-chain reaction found in genomics research[51], analyte concentrations are often low and detection methods need to be highly sensitive. In addition, analytical methods need to be of sufficient throughput to handle the samples sizes required to identify associations within the considerable population variance[49,50]. And, within these constraints, the methods need to have the actual capability to resolve the relevant glycosylation properties, which is complicated by glycan stereoisomerism and resulting numbers of chemically distinct structures that overlap in physical properties such as solubility, charge, and mass.

Glycoprotein analysis can in principle be performed at three levels, namely on intact glycoproteins, on glycopeptides, and on released glycans[44,52]. Intact glycoprotein analysis provides most information on the combination and interaction of glycans and other PTMs on a protein, approaching most closely the biological situation[53]. Proteolytic digestion of a glycoprotein into discrete peptides and glycopeptides, *e.g.*, by trypsin, GluC or proteinase K, may still retain the site-specific glycan information but reduces the analytical complexity to some degree[54]. Releasing glycans from the protein backbone, *e.g.*, by hydrazinolysis, β-elimination, or endoglycosidase digestion in case of *N*-glycosylation, can yield the most structural information on the glycan and has the highest tolerance to complex samples, but does not reveal the glycan attachment site or carrier protein[44,52,55,56].

Further distinction has to be made between the analysis of a single glycoprotein after targeted purification using, *e.g.*, affinity-capturing, and the analysis of a glycoprotein mixture. In-depth glycan analysis has been performed for a selection of single glycoproteins, examples including IgG, IgM, and alpha-1-antitrypsin[57-60], but these are limited by the availability of protein-specific purification protocols. For clinical biofluid glycosylation analysis, *e.g.*, serum or plasma, qualitative glycan information can be obtained as well, but this is usually restricted to the released glycan level due to the large array of glycoproteins involved[61,62]. These complex-sample released glycan analyses are generally referred to as "glycomics" experiments, and specific "glycomes" can be indicated by their sample source, *e.g.*, the total serum protein *N*-glycome (TSNG) or the total plasma protein *N*-glycome (TPNG). With the diversity of glycan structures involved in such samples, a combination of methods is typically required for complete structural characterization – each of the methods ideally providing orthogonal information[63].

It is to no surprise then that a broad selection of analytical methods have been applied to the characterization of glycans and glycoconjugates. These include, among others, lectin or carbohydrate microarrays[64,65], nuclear magnetic resonance spectroscopy[60,66], liquid chromatography[59,67], capillary electrophoresis (CE)[68,69], and mass spectrometry (MS)[61,70], as

well as a variety of hyphenations among them (*e.g.*, CE-MS or LC-MS)[63,71,72]. Large-scale *N*-glycomics studies have so far been reported with hydrophilic-interaction (ultra-)high-performance liquid chromatography with fluorescence detection (HILIC-(U)HPLC-FLD)[33,46,73], capillary gel electrophoresis with laser-induced fluorescence detection (CGE-LIF)[74], and matrix-assisted laser desorption/ionization (MALDI) MS[48,63,75].

1.6 Matrix-assisted laser desorption/ionization mass spectrometry

Mass spectrometry is an analytical technique to form gas-phase ions, separate them by mass per charge (*m/z*), and report on their relative abundances. Mass spectrometers usually consist of an ion source, a separation step, and a means of detecting the ions[76].

To generate gas-phase ions MS can make use of a large selection of ionization methods, but common variants for high-throughput glycan analysis are electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI)[76]. ESI requires analytes to be dissolved in a liquid, which is dispersed into aerosols by the presence of a nebulizing gas and the application of a high voltage[76]. Ions may directly evaporate from these aerosols or solvent evaporation may leave behind ionized residual molecules[76]. MALDI, on the other hand, requires a sample of interest to be co-crystallized with a matrix substance. Gaseous molecules are generated by irradiating the crystals with a laser, leading to sublimation of the molecules. In the gas-phase the analytes of interest are ionized by deprotonation, protonation, or alkali metal adduction[76]. Unlike ESI, MALDI predominantly generates single-charged ions, which is unfavorable for the detection of very large masses, but on the other hand also leads to relatively clean mass spectra in the *m/z* range covering released human *N*-glycosylation[77]. Compared to ESI, MALDI is an extremely high-throughput method for the ionization of successive samples, as these can be pre-spotted on a target plate and indexed within seconds from each other[63,76].

After ionization, separation of the gas-phase ions can be achieved in a variety of ways as well. For time-of-flight (TOF) MS the separation occurs by application of a static electric field. This provides greater acceleration to molecules that are lower in mass (*m*) and carry more charges (*z*, hence the *m/z* separation dimension), which is then followed by a drift time in a field-free region towards an ion detector[76]. Alternatively, ions can be trapped in an electric or magnetic field and[76,78], and for example be detected by their ion cyclotron resonance as is the case for Fourier transform ion cyclotron resonance (FTICR) MS[79,80].

Whereas MALDI-FTICR-MS can achieve very high resolution *N*-glycan measurements, potentially separating isobaric chemicals that are milliDaltons apart[79,80], MALDI-TOF-MS requires additional equipment to achieve isotopic resolution for *N*-glycan analysis[81,82]. One issue with MALDI is that molecules form a three-dimensional cloud prior to acceleration,

leading to different exposure times to the electric field for different regions of the cloud. This results in different kinetic energies for equivalent molecules, and ultimately a broader time range in which they arrive at the detector[81,82]. One way to solve the resulting peak broadening is by using a mass reflectron (ion mirror), which reflects the molecules in such a way that it corrects the kinetic energy range (**Figure 4**)[81,82]. In way of resolution, reflectron-mounted MALDI-TOF-MS instruments clearly outperform their linear counterparts. However, when molecules break down by metastable decay in the field-free region, reflection can lead to inaccuracy in both resolution and the perceived *m/z* value of the analyte (**Figure 5**)[77,83].

Table 1. *Monosaccharides detectable by mass spectrometry.*

Monosaccharide	Short	CFG Symbol	Letter	Increment composition	Increment mass
Deoxyhexose	**dHex**	△	**F**	**$C_6H_{10}O_4$**	**146.0579**
Fucose	Fuc	▲	F	$C_6H_{10}O_4$	146.0579
Hexose	**Hex**	○	**H**	**$C_6H_{10}O_5$**	**162.0528**
Glucose	Glc	●	H	$C_6H_{10}O_5$	162.0528
Mannose	Man	●	H	$C_6H_{10}O_5$	162.0528
Galactose	Gal	●	H	$C_6H_{10}O_5$	162.0528
***N*-Acetylhexosamine**	**HexNAc**	□	**N**	**$C_8H_{13}NO_5$**	**203.0794**
N-Acetylglucosamine	GlcNAc	■	N	$C_8H_{13}NO_5$	203.0794
N-Acetylgalactosamine	GalNAc	■	N	$C_8H_{13}NO_5$	203.0794
***N*-Acetylneuraminic acid**	**Neu5Ac**	◆	**S**	**$C_{11}H_{17}NO_8$**	**291.0954**
***N*-Glycolylneuraminic acid**	**Neu5Gc**	◇	**Gs**	**$C_{11}H_{17}NO_9$**	**307.0903**

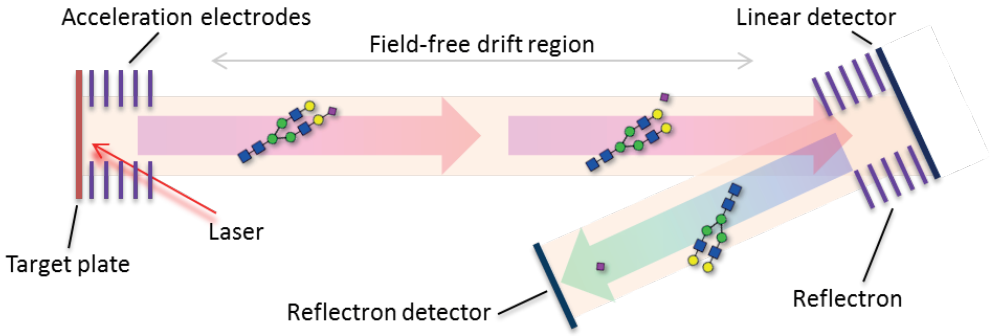

***Figure 4**. Scheme of a reflectron-mounted MALDI-TOF-MS system. Ions produced by MALDI are accelerated by a static electric field, further separated in the field-free drift region, and are either directly detected or after reflection to yield better resolution. In case an analyte breaks down by metastable decay reflection leads to inaccurate and imprecise signals.*

MALDI-MS has shown to be a powerful tool for the analysis of glycosylation, but several constraints apply as well[77]. As MS separation is primarily based on mass, different chemical compositions can usually be distinguished, but no information is obtained on the stereoisomeric aspects of a structure. As such, MS can define a glycan composition by the number of deoxyhexoses, hexoses and *N*-acetylhexosamines, but will not reveal whether these are derived from glucose, mannose or galactose, and in which linkages these are attached (**Table 1**)[77]. Sialic acids like *N*-acetylneuraminic acid and *N*-glycolylneuraminic acid can be distinguished by their unique masses as well, but analysis of sialylated glycans by MALDI-MS is challenging for other reasons. For one, while neutral glycans tend to ionize as cations together with sodium or potassium, sialylated glycans preferentially form anions by deprotonation of the carboxylic acids, which prevents their joint analysis when accelerated by a single polarity electric field[77]. Secondly, sialylated glycans are highly affected by in-source and metastable decay, meaning that mass spectra obtained by reflectron-mounted MALDI-TOF-MS are dominated by sialic acid loss and the appearance of unfocussed "metastable peaks" (**Figure 5**)[77,83].

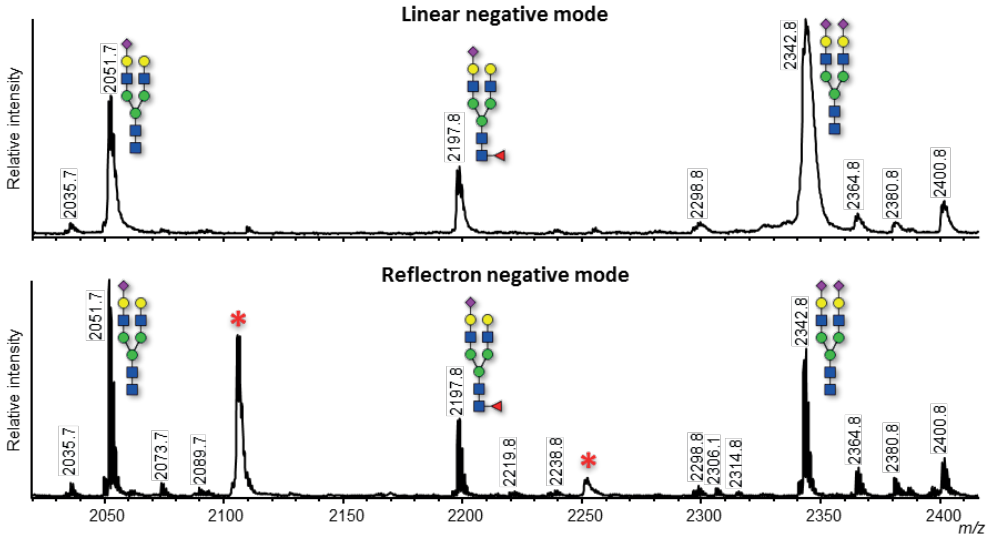

***Figure 5**. MALDI-TOF-MS profiles of 2-AA-labeled human plasma N-glycosylation in linear negative mode (**top**) and reflectron negative mode (**bottom**). Reflectron mode shows isotopic resolution whereas linear mode does not. However, reflectron mode shows the appearance of low-resolution "metastable peaks" (**asterisk**), at the expense of sialylated signals.*

1.7 Chemical derivatization for MALDI-MS glycan analysis

Without making use of derivatization, MALDI mass spectrometric experiments have tried to handle the analysis of sialylated glycan species in a variety of ways. Many reports have been made on the optimization of MALDI matrix conditions and constituents[77]. MALDI matrices such as 2,5-dihydroxybenzoic acid (2,5-DHB)[84], α-cyano-4-hydroxycinnamic acid (CHCA)[85],

or 5-chloro-α-cyanocinnamic acid (Cl-CCA)[86], can for instance be classified into "hot" and "cold" variants, which roughly describes the amount of energy they typically convey to their co-crystallized analytes of interest during the desorption / ionization process[77]. A matrix classified as "hot" allows easier ionization and fragmentation than "cold" matrices, but also leads to more in-source and metastable decay. However, no matrix has been described to date that fully prevents sialic acid decay.

Another way to improve the mass spectrometric detection of (sialylated) glycan species is the chemical alteration of problematic functional groups, and a variety of derivatization strategies have been reported over the years[56,75,77]. One popular method is permethylation, the conversion of a broad array of functional groups across a glycan into their methyl derivatives[55,87]. Permethylation amends the unfavorable ionization and stability situation of sialylated species and has proven to enhance positive ionization of carbohydrates in general[55,87]. A downside of permethylation is that even small reaction inefficiencies per functional group lead to significant peak splitting for larger glycan species. Another issue is the application of relatively harsh reaction conditions leading to sialic acid de-*O*-acetylation, an important modification which can occur on the hydroxyl groups of sialic acids[18,88,89]. Many studies have been published making use of glycan permethylation, both of *O*-glycans and *N*-glycans, and in general the derivatization is considered to be the baseline procedure for mass spectrometric glycan analysis[61,90-93].

Rather than chemical alteration of the wide variety of functional groups on a glycan, it also proved possible to specifically modify the carboxylic acids of the sialylated glycans, which tackled the ionization and stability issues as well. Workflows have been presented that rely on sialic acid esterification or amidation, encompassing a wide variety of acid activator reagents and nucleophiles to arrive at stabilized and neutralized *N*-glycans[56,75,77], several of which have proven viable for the chemical modification of glycopeptides as well[94-97].

Interestingly, the investigations into sialic acid derivatization have led to the observation that α2,3-linked *N*-acetylneuraminic acids – and not α2,6-linked *N*-acetylneuraminic acids – tend to lose water under harsh reaction conditions, understood to be lactonization with the subterminal galactose[98,99]. The first chemistry to exploit this linkage-specific modification for *N*-glycan analysis was with the use of the carboxylic acid activator DMT-MM, repeatedly added in crystalline form to a methanol solution with dissolved glycans[99]. As described, performing the reaction for 1 hour at 80 °C, or 6 hours at room temperature, led to the methyl esterification of α2,6-linked *N*-acetylneuraminic acids, and could also catalyze the lactonization of α2,3-linked sialic acids[99]. Next to neutralizing and stabilizing the sialylated glycans for MS analysis, the mass difference resulting from the linkage-specific reaction allowed mass-based distinction of their stereoisomerism[99].

Following a similar strategy, we showed that linkage-specific sialic acid esterification was as well achievable as a high-throughput one-pot reaction, with increased speed and at milder reaction conditions (1 hour at 37 °C proved sufficient in all cases), by making use of the carboxylic acid activator EDC and catalyst HOBt in an environment of ethanol[62,100]. For this reaction, the use of ethanol instead of methanol proved a requirement for the full linkage-specificity, consequentially leading to linkage-dependent ethyl esterification or lactonization[62,100].

1.8 Scope

The aim of this thesis is the development and application of new methodology for MALDI-MS *N*-glycomics, in order to improve our detection of glycosylation characteristics in a high-throughput manner, and to increase our understanding of the role of *N*-glycosylation in health and disease.

Part I introduces the field of research, and contains the general introduction (**Chapter 1**), as well as a literature review on high-throughput *N*-glycosylation analysis (**Chapter 2**).

Presented in **Part II** are the methodological advances that were achieved in glycosylation analysis by MALDI-TOF-MS. **Chapter 3** reports on a high-throughput workflow for the derivatization of sialylated *N*-glycans, allowing the concurrent detection of sialylated and non-sialylated glycan species in reflectron positive mode MALDI-TOF-MS, as well introducing a distinguishable mass difference between α2,3- and α2,6-linked sialylation isomers. This method was extended to an automated platform in **Chapter 4**.

Part III describes the application of the protocols in settings of clinical and biopharmaceutical interest. Much of current-day biopharmaceutical and fundamental research is performed on experimental animal models, of which the mouse is an abundant example. The plasma *N*-glycosylation of different mouse strains is reported in **Chapter 5**, as well as the differences between male and female individuals of those strains. In **Chapter 6** an alternative sialic acid protection method for MALDI-MS is described, namely the application of collisional cooling in an intermediate-pressure-sourced MALDI-FTICR-MS. This has led to the largest mass spectrometric TPNG study to date, and shows the association between *N*-glycan phenotypes and blood markers of inflammation and metabolic health. In **Chapter 7** the robotized MALDI-TOF-MS method is applied to study – in longitudinal fashion – the glycosylation parameters reflective of RA and the improvement of disease activity thereof during pregnancy. Finally, **Chapter 8** describes a collaboration with several leading high-throughput glycomics laboratories to compare the performance of the contemporary serum *N*-glycomics methodologies, *i.e.*, HILIC-UHPLC-FLD, multiplexed CGE-LIF, and MALDI-TOF-MS with ethyl esterification.

Part IV contains the general discussion (**Chapter 9**). This chapter puts the performed research in the literature context, and speculates on the requirements and challenges for the future of mass spectrometric *N*-glycosylation analysis.

1.9 References

1 Apweiler, R., Hermjakob, H. & Sharon, N. On the frequency of protein glycosylation, as deduced from analysis of the SWISS-PROT database. *Biochim Biophys Acta* **1473**, 4-8 (1999).

2 Khoury, G. A., Baliban, R. C. & Floudas, C. A. Proteome-wide post-translational modification statistics: frequency analysis and curation of the swiss-prot database. *Sci Rep* **1**, doi:10.1038/srep00090 (2011).

3 Varki, A. *et al. Essentials of Glycobiology*. 2nd edn, (2009).

4 Culyba, E. K. *et al.* Protein native-state stabilization by placing aromatic side chains in N-glycosylated reverse turns. *Science* **331**, 571-575, doi:10.1126/science.1198461 (2011).

5 Caramelo, J. J. & Parodi, A. J. A sweet code for glycoprotein folding. *FEBS Lett* **589**, 3379-3387, doi:10.1016/j.febslet.2015.07.021 (2015).

6 Roth, J. *et al.* Protein N-glycosylation, protein folding, and protein quality control. *Mol Cells* **30**, 497-506, doi:10.1007/s10059-010-0159-z (2010).

7 Duman, J. G. Animal ice-binding (antifreeze) proteins and glycolipids: an overview with emphasis on physiological function. *J Exp Biol* **218**, 1846-1855, doi:10.1242/jeb.116905 (2015).

8 Vaishnava, S. *et al.* The antibacterial lectin RegIIIgamma promotes the spatial segregation of microbiota and host in the intestine. *Science* **334**, 255-258, doi:10.1126/science.1209791 (2011).

9 Sharon, N. & Lis, H. History of lectins: from hemagglutinins to biological recognition molecules. *Glycobiology* **14**, 53R-62R, doi:10.1093/glycob/cwh122 (2004).

10 Mimura, Y. *et al.* Glycosylation engineering of therapeutic IgG antibodies: challenges for the safety, functionality and efficacy. *Protein Cell*, doi:10.1007/s13238-017-0433-3 (2017).

11 Varki, A. Biological roles of glycans. *Glycobiology* **27**, 3-49, doi:10.1093/glycob/cww086 (2017).

12 Moremen, K. W., Tiemeyer, M. & Nairn, A. V. Vertebrate protein glycosylation: diversity, synthesis and function. *Nat Rev Mol Cell Biol* **13**, 448-462, doi:10.1038/nrm3383 (2012).

13 Kaplan, H. A., Welply, J. K. & Lennarz, W. J. Oligosaccharyl transferase: the central enzyme in the pathway of glycoprotein assembly. *Biochim Biophys Acta* **906**, 161-173 (1987).

14 Van den Steen, P., Rudd, P. M., Dwek, R. A. & Opdenakker, G. Concepts and principles of O-linked glycosylation. *Crit Rev Biochem Mol Biol* **33**, 151-208, doi:10.1080/10409239891204198 (1998).

15 Spiro, R. G. Protein glycosylation: nature, distribution, enzymatic formation, and disease implications of glycopeptide bonds. *Glycobiology* **12**, 43R-56R (2002).

16 Amado, M., Almeida, R., Schwientek, T. & Clausen, H. Identification and characterization of large galactosyltransferase gene families: galactosyltransferases for all functions. *Biochim Biophys Acta* **1473**, 35-53 (1999).

17 Laine, R. A. A calculation of all possible oligosaccharide isomers both branched and linear yields 1.05 x 10(12) structures for a reducing hexasaccharide: the Isomer Barrier to development of single-method saccharide sequencing or synthesis systems. *Glycobiology* **4**, 759-767 (1994).

18 Varki, A. Diversity in the sialic acids. *Glycobiology* **2**, 25-40 (1992).

19 Varki, A. *et al.* Symbol Nomenclature for Graphical Representations of Glycans. *Glycobiology* **25**, 1323-1324, doi:10.1093/glycob/cwv091 (2015).

20 Smit, C. H. *et al.* Surface expression patterns of defined glycan antigens change during Schistosoma mansoni cercarial transformation and development of schistosomula. *Glycobiology* **25**, 1465-1479, doi:10.1093/glycob/cwv066 (2015).
21 Ellgaard, L. & Helenius, A. Quality control in the endoplasmic reticulum. *Nat Rev Mol Cell Biol* **4**, 181-191, doi:10.1038/nrm1052 (2003).
22 Tyrrell, D. *et al.* Structural requirements for the carbohydrate ligand of E-selectin. *Proc Natl Acad Sci U S A* **88**, 10372-10376 (1991).
23 Nairn, A. V. *et al.* Regulation of glycan structures in animal tissues: transcript profiling of glycan-related genes. *J Biol Chem* **283**, 17298-17313, doi:10.1074/jbc.M801964200 (2008).
24 Cummings, R. D. The repertoire of glycan determinants in the human glycome. *Mol Biosyst* **5**, 1087-1104, doi:10.1039/b907931a (2009).
25 Freeze, H. H. Genetic defects in the human glycome. *Nat Rev Genet* **7**, 537-551, doi:10.1038/nrg1894 (2006).
26 Niehues, R. *et al.* Carbohydrate-deficient glycoprotein syndrome type Ib. Phosphomannose isomerase deficiency and mannose therapy. *J Clin Invest* **101**, 1414-1420, doi:10.1172/JCI2350 (1998).
27 Marquardt, T. *et al.* Correction of leukocyte adhesion deficiency type II with oral fucose. *Blood* **94**, 3976-3985 (1999).
28 Tegtmeyer, L. C. *et al.* Multiple phenotypes in phosphoglucomutase 1 deficiency. *N Engl J Med* **370**, 533-542, doi:10.1056/NEJMoa1206605 (2014).
29 Pinho, S. S. & Reis, C. A. Glycosylation in cancer: mechanisms and clinical implications. *Nat Rev Cancer* **15**, 540-555, doi:10.1038/nrc3982 (2015).
30 Meany, D. L., Zhang, Z., Sokoll, L. J., Zhang, H. & Chan, D. W. Glycoproteomics for prostate cancer detection: changes in serum PSA glycosylation patterns. *J Proteome Res* **8**, 613-619, doi:10.1021/pr8007539 (2009).
31 Jorgensen, T. *et al.* Up-regulation of the oligosaccharide sialyl LewisX: a new prognostic parameter in metastatic prostate cancer. *Cancer Res* **55**, 1817-1819 (1995).
32 Thanabalasingham, G. *et al.* Mutations in HNF1A result in marked alterations of plasma glycan profile. *Diabetes* **62**, 1329-1337, doi:10.2337/db12-0880 (2013).
33 Lauc, G. *et al.* Genomics meets glycomics-the first GWAS study of human N-Glycome identifies HNF1alpha as a master regulator of plasma protein fucosylation. *PLoS Genet* **6**, e1001256, doi:10.1371/journal.pgen.1001256 (2010).
34 Arnold, J. N., Wormald, M. R., Sim, R. B., Rudd, P. M. & Dwek, R. A. The impact of glycosylation on the biological function and structure of human immunoglobulins. *Annu Rev Immunol* **25**, 21-50, doi:10.1146/annurev.immunol.25.022106.141702 (2007).
35 Holland, M. *et al.* Differential glycosylation of polyclonal IgG, IgG-Fc and IgG-Fab isolated from the sera of patients with ANCA-associated systemic vasculitis. *Biochim Biophys Acta* **1760**, 669-677, doi:10.1016/j.bbagen.2005.11.021 (2006).
36 Shields, R. L. *et al.* Lack of fucose on human IgG1 N-linked oligosaccharide improves binding to human Fcgamma RIII and antibody-dependent cellular toxicity. *J Biol Chem* **277**, 26733-26740, doi:10.1074/jbc.M202069200 (2002).
37 Thomann, M. *et al.* In vitro glycoengineering of IgG1 and its effect on Fc receptor binding and ADCC activity. *PLoS One* **10**, e0134949, doi:10.1371/journal.pone.0134949 (2015).
38 Thomann, M., Reckermann, K., Reusch, D., Prasser, J. & Tejada, M. L. Fc-galactosylation modulates antibody-dependent cellular cytotoxicity of therapeutic antibodies. *Mol Immunol* **73**, 69-75, doi:10.1016/j.molimm.2016.03.002 (2016).
39 Albrecht, S., Unwin, L., Muniyappa, M. & Rudd, P. M. Glycosylation as a marker for inflammatory arthritis. *Cancer Biomark* **14**, 17-28, doi:10.3233/CBM-130373 (2014).
40 van de Geijn, F. E. *et al.* Immunoglobulin G galactosylation and sialylation are associated with pregnancy-induced improvement of rheumatoid arthritis and the postpartum flare:

results from a large prospective cohort study. *Arthritis Res Ther* **11**, R193, doi:10.1186/ar2892 (2009).
41 Bondt, A. *et al.* Association between galactosylation of immunoglobulin G and improvement of rheumatoid arthritis during pregnancy is independent of sialylation. *J Proteome Res* **12**, 4522-4531, doi:10.1021/pr400589m (2013).
42 Hafkenscheid, L. *et al.* Structural Analysis of Variable Domain Glycosylation of Anti-Citrullinated Protein Antibodies in Rheumatoid Arthritis Reveals the Presence of Highly Sialylated Glycans. *Mol Cell Proteomics* **16**, 278-287, doi:10.1074/mcp.M116.062919 (2017).
43 Reusch, D. & Tejada, M. L. Fc glycans of therapeutic antibodies as critical quality attributes. *Glycobiology* **25**, 1325-1334, doi:10.1093/glycob/cwv065 (2015).
44 Zauner, G. *et al.* Glycoproteomic analysis of antibodies. *Mol Cell Proteomics* **12**, 856-865, doi:10.1074/mcp.R112.026005 (2013).
45 Knezevic, A. *et al.* Effects of aging, body mass index, plasma lipid profiles, and smoking on human plasma N-glycans. *Glycobiology* **20**, 959-969, doi:10.1093/glycob/cwq051 (2010).
46 Ruhaak, L. R. *et al.* Plasma protein N-glycan profiles are associated with calendar age, familial longevity and health. *J Proteome Res* **10**, 1667-1674, doi:10.1021/pr1009959 (2011).
47 Jansen, B. C. *et al.* Pregnancy-associated serum N-glycome changes studied by high-throughput MALDI-TOF-MS. *Sci Rep* **6**, 23296, doi:10.1038/srep23296 (2016).
48 Reiding, K. R. *et al.* Human Plasma N-glycosylation as Analyzed by Matrix-Assisted Laser Desorption/Ionization-Fourier Transform Ion Cyclotron Resonance-MS Associates with Markers of Inflammation and Metabolic Health. *Mol Cell Proteomics* **16**, 228-242, doi:10.1074/mcp.M116.065250 (2017).
49 Gornik, O. *et al.* Stability of N-glycan profiles in human plasma. *Glycobiology* **19**, 1547-1553, doi:10.1093/glycob/cwp134 (2009).
50 Hennig, R. *et al.* Towards personalized diagnostics via longitudinal study of the human plasma N-glycome. *Biochim Biophys Acta* **1860**, 1728-1738, doi:10.1016/j.bbagen.2016.03.035 (2016).
51 Saiki, R. K. *et al.* Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **239**, 487-491 (1988).
52 Geyer, H. & Geyer, R. Strategies for analysis of glycoprotein glycosylation. *Bba-Proteins Proteom* **1764**, 1853-1869, doi:10.1016/j.bbapap.2006.10.007 (2006).
53 Thompson, N. J., Rosati, S., Rose, R. J. & Heck, A. J. The impact of mass spectrometry on the study of intact antibodies: from post-translational modifications to structural analysis. *Chem Commun (Camb)* **49**, 538-548, doi:10.1039/c2cc36755f (2013).
54 Dalpathado, D. S. & Desaire, H. Glycopeptide analysis by mass spectrometry. *The Analyst* **133**, 731-738, doi:10.1039/b713816d (2008).
55 Morelle, W. & Michalski, J. C. Analysis of protein glycosylation by mass spectrometry. *Nat Protoc* **2**, 1585-1602, doi:10.1038/nprot.2007.227 (2007).
56 Gaunitz, S., Nagy, G., Pohl, N. L. & Novotny, M. V. Recent Advances in the Analysis of Complex Glycoproteins. *Anal Chem* **89**, 389-413, doi:10.1021/acs.analchem.6b04343 (2017).
57 Reusch, D. *et al.* Comparison of methods for the analysis of therapeutic immunoglobulin G Fc-glycosylation profiles--part 1: separation-based methods. *MAbs* **7**, 167-179, doi:10.4161/19420862.2014.986000 (2015).
58 Reusch, D. *et al.* Comparison of methods for the analysis of therapeutic immunoglobulin G Fc-glycosylation profiles-Part 2: Mass spectrometric methods. *MAbs* **7**, 732-742, doi:10.1080/19420862.2015.1045173 (2015).

59 Arnold, J. N. *et al.* Human serum IgM glycosylation - Identification of glycoforms that can bind to mannan-binding lectin. *Journal of Biological Chemistry* **280**, 29080-29087, doi:10.1074/jbc.M504528200 (2005).
60 Kolarich, D., Weber, A., Turecek, P. L., Schwarz, H. P. & Altmann, F. Comprehensive glyco-proteomic analysis of human alpha1-antitrypsin and its charge isoforms. *Proteomics* **6**, 3369-3380, doi:10.1002/pmic.200500751 (2006).
61 Stumpo, K. A. & Reinhold, V. N. The N-glycome of human plasma. *J Proteome Res* **9**, 4823-4830, doi:10.1021/pr100528k (2010).
62 Reiding, K. R., Blank, D., Kuijper, D. M., Deelder, A. M. & Wuhrer, M. High-throughput profiling of protein N-glycosylation by MALDI-TOF-MS employing linkage-specific sialic acid esterification. *Anal Chem* **86**, 5784-5793, doi:10.1021/ac500335t (2014).
63 Huffman, J. E. *et al.* Comparative performance of four methods for high-throughput glycosylation analysis of immunoglobulin G in genetic and epidemiological research. *Mol Cell Proteomics* **13**, 1598-1610, doi:10.1074/mcp.M113.037465 (2014).
64 Etxebarria, J., Calvo, J., Martin-Lomas, M. & Reichardt, N. C. Lectin-array blotting: profiling protein glycosylation in complex mixtures. *ACS Chem Biol* **7**, 1729-1737, doi:10.1021/cb300262x (2012).
65 de Boer, A. R., Hokke, C. H., Deelder, A. M. & Wuhrer, M. General microarray technique for immobilization and screening of natural glycans. *Anal Chem* **79**, 8107-8113, doi:10.1021/ac071187g (2007).
66 Stroop, C. J. *et al.* Characterization of the carbohydrate chains of the secreted form of the human epidermal growth factor receptor. *Glycobiology* **10**, 901-917 (2000).
67 Bones, J., Mittermayr, S., O'Donoghue, N., Guttman, A. & Rudd, P. M. Ultra performance liquid chromatographic profiling of serum N-glycans for fast and efficient identification of cancer associated alterations in glycosylation. *Anal Chem* **82**, 10208-10215, doi:10.1021/ac102860w (2010).
68 Callewaert, N., Geysens, S., Molemans, F. & Contreras, R. Ultrasensitive profiling and sequencing of N-linked oligosaccharides using standard DNA-sequencing equipment. *Glycobiology* **11**, 275-281 (2001).
69 Ruhaak, L. R. *et al.* Optimized workflow for preparation of APTS-labeled N-glycans allowing high-throughput analysis of human plasma glycomes using 48-channel multiplexed CGE-LIF. *J Proteome Res* **9**, 6655-6664, doi:10.1021/pr100802f (2010).
70 Goetz, J. A., Mechref, Y., Kang, P., Jeng, M. H. & Novotny, M. V. Glycomic profiling of invasive and non-invasive breast cancer cells. *Glycoconj J* **26**, 117-131, doi:10.1007/s10719-008-9170-4 (2009).
71 Khatri, K. *et al.* Microfluidic Capillary Electrophoresis-Mass Spectrometry for Analysis of Monosaccharides, Oligosaccharides, and Glycopeptides. *Anal Chem* **89**, 6645-6655, doi:10.1021/acs.analchem.7b00875 (2017).
72 Reusch, D. *et al.* High-throughput work flow for IgG Fc-glycosylation analysis of biotechnological samples. *Anal Biochem* **432**, 82-89, doi:10.1016/j.ab.2012.09.032 (2013).
73 Lauc, G. *et al.* Loci associated with N-glycosylation of human immunoglobulin G show pleiotropy with autoimmune diseases and haematological cancers. *PLoS Genet* **9**, e1003225, doi:10.1371/journal.pgen.1003225 (2013).
74 Ruhaak, L. R. *et al.* Targeted biomarker discovery by high throughput glycosylation profiling of human plasma alpha1-antitrypsin and immunoglobulin A. *PLoS One* **8**, e73082, doi:10.1371/journal.pone.0073082 (2013).
75 Shubhakar, A. *et al.* High-Throughput Analysis and Automation for Glycomics Studies. *Chromatographia* **78**, 321-333, doi:10.1007/s10337-014-2803-9 (2015).
76 de Hoffmann, E. & Stroobant, V. *Mass spectrometry: principles and applications*. 3rd edn, (2007).

77 Harvey, D. J. Matrix-assisted laser desorption/ionization mass spectrometry of carbohydrates. *Mass Spectrom Rev* **18**, 349-450, doi:10.1002/(SICI)1098-2787(1999)18:6<349::AID-MAS1>3.0.CO;2-H (1999).
78 Blaum, K. High-accuracy mass spectrometry with stored ions. *Phys Rep* **425**, 1-78, doi:10.1016/j.physrep.2005.10.011 (2006).
79 Marshall, A. G., Hendrickson, C. L. & Jackson, G. S. Fourier transform ion cyclotron resonance mass spectrometry: A primer. *Mass Spectrom Rev* **17**, 1-35, doi:Doi 10.1002/(Sici)1098-2787(1998)17:1<1::Aid-Mas1>3.0.Co;2-K (1998).
80 Zhang, L. K., Rempel, D., Pramanik, B. N. & Gross, M. L. Accurate mass measurements by Fourier transform mass spectrometry. *Mass Spectrom Rev* **24**, 286-309, doi:10.1002/mas.20013 (2005).
81 Berger, C. Compensator Role of the Electrostatic Mirror in Time of Flight Mass-Spectrometry. *International Journal of Mass Spectrometry and Ion Processes* **46**, 63-66, doi:Doi 10.1016/0020-7381(83)80053-9 (1983).
82 Mamyrin, B. A. Time-of-flight mass spectrometry (concepts, achievements, and prospects). *Int J Mass Spectrom* **206**, 251-266, doi:Doi 10.1016/S1387-3806(00)00392-4 (2001).
83 Harvey, D. J., Hunter, A. P., Bateman, R. H., Brown, J. & Critchley, G. Relationship between in-source and post-source fragment ions in the matrix-assisted laser desorption (ionization) mass spectra of carbohydrates recorded with reflectron time-of-flight mass spectrometers. *Int J Mass Spectrom* **188**, 131-146, doi:Doi 10.1016/S1387-3806(99)00014-7 (1999).
84 Stahl, B., Steup, M., Karas, M. & Hillenkamp, F. Analysis of Neutral Oligosaccharides by Matrix-Assisted Laser Desorption - Ionization Mass-Spectrometry. *Anal Chem* **63**, 1463-1466, doi:Doi 10.1021/Ac00014a022 (1991).
85 Beavis, R. C., Chaudhary, T. & Chait, B. T. Alpha-Cyano-4-Hydroxycinnamic Acid as a Matrix for Matrix-Assisted Laser Desorption Mass-Spectrometry. *Org Mass Spectrom* **27**, 156-158, doi:DOI 10.1002/oms.1210270217 (1992).
86 Selman, M. H. *et al.* MALDI-TOF-MS analysis of sialylated glycans and glycopeptides using 4-chloro-alpha-cyanocinnamic acid matrix. *Proteomics* **12**, 1337-1348, doi:10.1002/pmic.201100498 (2012).
87 Ciucanu, I. & Kerek, F. A Simple and Rapid Method for the Permethylation of Carbohydrates. *Carbohyd Res* **131**, 209-217, doi:Doi 10.1016/0008-6215(84)85242-8 (1984).
88 Wu, Z. *et al.* Characterization of O-acetylation in sialoglycans by MALDI-MS using a combination of methylamidation and permethylation. *Sci Rep* **7**, 46206, doi:10.1038/srep46206 (2017).
89 Klein, A. & Roussel, P. O-acetylation of sialic acids. *Biochimie* **80**, 49-57 (1998).
90 Kang, P., Mechref, Y. & Novotny, M. V. High-throughput solid-phase permethylation of glycans prior to mass spectrometry. *Rapid Commun Mass Spectrom* **22**, 721-734, doi:10.1002/rcm.3395 (2008).
91 Shubhakar, A. *et al.* Automated High-Throughput Permethylation for Glycosylation Analysis of Biologics Using MALDI-TOF-MS. *Anal Chem* **88**, 8562-8569, doi:10.1021/acs.analchem.6b01639 (2016).
92 Parry, S. *et al.* The sperm agglutination antigen-1 (SAGA-1) glycoforms of CD52 are O-glycosylated. *Glycobiology* **17**, 1120-1126, doi:10.1093/glycob/cwm076 (2007).
93 Boztug, K. *et al.* JAGN1 deficiency causes aberrant myeloid cell homeostasis and congenital neutropenia. *Nat Genet* **46**, 1021-+, doi:10.1038/ng.3069 (2014).
94 Nishikaze, T., Nakamura, T., Jinmei, H. & Amano, J. Negative-ion MALDI-MS2 for discrimination of alpha2,3- and alpha2,6-sialylation on glycopeptides labeled with a pyrene derivative. *J Chromatogr B Analyt Technol Biomed Life Sci* **879**, 1419-1428, doi:10.1016/j.jchromb.2010.10.032 (2011).

95 Amano, J. *et al.* Derivatization with 1-pyrenyldiazomethane enhances ionization of glycopeptides but not peptides in matrix-assisted laser desorption/ionization mass spectrometry. *Anal Chem* **82**, 8738-8743, doi:10.1021/ac101555a (2010).

96 de Haan, N. *et al.* Linkage-specific sialic acid derivatization for MALDI-TOF-MS profiling of IgG glycopeptides. *Anal Chem* **87**, 8284-8291, doi:10.1021/acs.analchem.5b02426 (2015).

97 Gomes de Oliveira, A. G. *et al.* A systematic study of glycopeptide esterification for the semi-quantitative determination of sialylation in antibodies. *Rapid Commun Mass Spectrom* **29**, 1817-1826, doi:10.1002/rcm.7287 (2015).

98 Pudelko, M. *et al.* Formation of lactones from sialylated MUC1 glycopeptides. *Org Biomol Chem* **4**, 713-720, doi:10.1039/b514918e (2006).

99 Wheeler, S. F., Domann, P. & Harvey, D. J. Derivatization of sialic acids for stabilization in matrix-assisted laser desorption/ionization mass spectrometry and concomitant differentiation of alpha(2 --> 3)- and alpha(2 --> 6)-isomers. *Rapid Commun Mass Spectrom* **23**, 303-312, doi:10.1002/rcm.3867 (2009).

100 Reiding, K. R., Lonardi, E., Hipgrave Ederveen, A. L. & Wuhrer, M. Ethyl Esterification for MALDI-MS Analysis of Protein Glycosylation. *Methods Mol Biol* **1394**, 151-162, doi:10.1007/978-1-4939-3341-9_11 (2016).