# Clinical glycomics of antibodies by mass spectometry

Haan, N. de

Chapter 2

# Linkage-Specific Sialic Acid Derivatization for MALDI-TOF-MS Profiling of IgG Glycopeptides

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Glycosylation is a common co- and post-translational protein modification, having a large influence on protein properties like conformation and solubility. Furthermore, glycosylation is an important determinant of efficacy and clearance of biopharmaceuticals such as immunoglobulin G (IgG). Matrix-assisted laser desorption/ionization (MALDI)-time of flight (TOF)-mass spectrometry (MS) shows potential for the site-specific glycosylation analysis of IgG at the glycopeptide level. With this approach however, important information about glycopeptide sialylation is not duly covered because of in-source and metastable decay of the sialylated species.

Here, we present a highly repeatable sialic acid derivatization method to allow subclass-specific MALDI-TOF-MS analysis of tryptic IgG glycopeptides. The method, employing dimethylamidation with the carboxylic acid activator 1-ethyl-3-(3-dimethylamino)propyl)-carbodiimide (EDC) and the catalyst 1-hydroxybenzotriazole (HOBt), results in different masses for the functionally divergent α2,3- and α2,6-linked sialic acids. Respective lactonization and dimethylamidation leads to their direct discrimination in MS and importantly, both glycan and peptide moieties reacted in a controlled manner. In addition, stabilization allowed the acquisition of fragmentation spectra informative with respect to glycosylation and peptide sequence. This was in contrast to fragmentation spectra of underivatized samples which were dominated by sialic acid loss. The method allowed the facile discrimination and relative quantitation of IgG Fc sialylation in therapeutic IgG samples. The method has considerable potential for future site- and sialic acid linkage-specific glycosylation profiling of therapeutic antibodies, as well as for subclass-specific biomarker discovery in clinical IgG samples derived from plasma.

## 2.1 Introduction

Glycosylation is an important and prevalent co- and post-translational protein modification, affecting the physiological and biochemical properties of the conjugate in numerous ways ranging from changes in solubility and half-life to modulation of receptor interaction [150-153]. Protein properties vary not only by glycosylation site occupancy, but also by the type of glycan present on a specific site, and while mammalian glycans are composed of a limited number of monosaccharides, the way in which the monosaccharides can be linked and branched adds considerable complexity to a molecule [154]. One notable example of this is *N*-acetylneuraminic acid. This sugar residue often terminates complex glycan antennae and can be linked either via an α2,6- or an α2,3-linkage to the subterminal galactose. On intravenous immunoglobulins (IVIG), the presence of an α2,6-linked sialic acid reduces inflammation, whereas an α2,3-linkage does not have this effect [155]. For a commonly used biopharmaceutical protein like immunoglobulin G (IgG), careful analysis and control of glycosylation is a prerequisite to ensure proper conformation, activity and clearance [156-159].

High-throughput analysis of protein glycosylation can be performed by mass spectrometry (MS), matrix-assisted laser desorption/ionization (MALDI)-time of flight (TOF)-MS being a particularly suitable approach [160, 161]. Using this method however, information on monosaccharide linkages is often difficult to obtain, and the analysis of sialylated glycan species is biased by metastable decay and variations in ionization and salt adduction [118, 160]. Derivatization methods are known for released glycans that allow for sialic acid stabilization in MALDI-MS as well as discrimination of sialylation linkage isomers in MS [123, 162]. Previously, we have reported ethyl esterification for this purpose, which is characterized by its facile workflow and high linkage-specificity [29, 124]. The protocol relies on the tendency of α2,3-linked sialic acids to lactonize, whereas α2,6-linked sialic acids are more susceptible to reactions with external alcohols or amines. However, as the protocol requires prior release of the glycans from the protein backbone, site-specificity, as well as subclass-specificity in case of IgG, is lost. To retain this information we focus here on the analysis of glycopeptides, which has shown its importance in glycosylation analysis. Notable examples of this include subclass-specific IgG glycosylation profiling and system-wide protein glycosylation mapping [163, 164]. In the case of IgG, glycopeptide analysis makes it possible to differentiate between Fab and Fc glycosylation, which is, amongst others, important in lectin binding studies [29]. Furthermore, because the analysis of glycopeptides gives protein specific information, it can be performed on relatively impure samples. In literature, efforts to stabilize sialylated glycopeptides have included complete methylamidation of all carboxylic acids present on the conjugate using (7-azabenzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate as coupling reagent, as well as a direct esterification by 1-pyrenyldiazomethane [128, 165, 166]. While

these approaches have indeed shown improved sialylation analysis by MS, and can achieve recognition of sialic acid linkage upon fragmentation, no report has been made of a glycopeptide derivatization that reacts in a selective way to differently linked sialic acids.

Here, we present a method for the sialic acid linkage-specific stabilization of IgG glycopeptides, which is able to derivate both *N*-acetylneuraminic acid and *N*-glycolylneuraminic acid and is therefore applicable on samples derived from various sources. Similar to the ethyl esterification reported previously, the method makes use of the carboxylic acid activator 1-ethyl-3-(3-dimethylamino)propyl)carbodiimide (EDC) and the catalyst 1-hydroxybenzotriazole (HOBt) [124]. However, we found that the alcohols used for the previously reported esterification, while linkage-specific for the sialic acid, showed variable reactions on the peptide portion of the glycoconjugate. Testing a range of reagents and conditions yielded a protocol employing dimethylamidation of the glycopeptides, which shows selectivity for the sialic acid linkage as well as for the carboxylic acids on the peptide portion. The protocol proves highly repeatable for the subclass-specific glycosylation analysis of tryptic IgG glycopeptides and provides, next to the sialic acid linkage information directly in MS, the possibility to generate informative MS/MS spectra. Finally, for two monoclonal antibodies with various sialic acid linkages, the profile of the glycopeptides after derivatization was found to be comparable to the analysis of released glycans. The speed and simplicity of the here reported method distinguishes it from other site- and sialic acid-specific methods like LC-MS, capillary electrophoresis (CE)-MS and the use of sialidases.

## 2.2 Experimental section

### 2.2.1 Chemicals, reagents, enzymes

The ultrapure deionized water (MQ) used in this study was generated from a Q-Gard 2 system (Millipore, Amsterdam, Netherlands), maintained at ≥18 MΩ. Ethanol (EtOH), methanol (MeOH), trifluoroacetic acid (TFA), sodium dodecyl sulfate (SDS), disodium hydrogen phosphate dihydrate ($Na_2HPO_4 \cdot 2H_2O$), potassium dihydrogen phosphate ($KH_2PO_4$) and sodium chloride (NaCl) were purchased from Merck (Darmstadt, Germany). TPCK treated trypsin (from bovine pancreas), HOBt hydrate, dimethyl sulfoxide (DMSO), 50% sodium hydroxide (NaOH), 40% dimethylamine in water, super-DHB, Nonidet P-40 (NP-40), sodium bicarbonate and formic acid (FA) were all purchased from Sigma-Aldrich (Steinheim, Germany) and EDC hydrochloride from Fluorochem (Hadfield, UK). Peptide-*N*-glycosidase F (PNGase F) was acquired from Roche Diagnostics (Mannheim, Germany), 2,5-dihydroxybenzoic acid (2,5-DHB) from Bruker Daltonics (Bremen, Germany), 4-chloro-α-cyanocinnamic acid (Cl-CCA) from Bionet Research (Camelford, Cornwall, UK) and HPLC SupraGradient acetonitrile (ACN) from Biosolve (Valkenswaard, Netherlands). 10x

Phosphate-buffered saline (10x PBS) was made in-house, containing 57 g/L $Na_2HPO_4 \cdot 2H_2O$, 5 g/L $KH_2PO_4$ and 85 g/L NaCl.

### 2.2.2 Samples

The IgG1 standard from human plasma was acquired from Athens Research (Athens, GA) and IgG from bovine serum from Sigma-Aldrich (Steinheim, Germany). Monoclonal antibodies originating from CHO cells (mAb1), as well as an *in vitro* galactosyl- and sialyltransferase treated variant thereof (mAb2) were provided by Roche Diagnostics. More details about mAb2 production can be found in **S-1.1** and **S-1.2**, Supporting Information. CHO cell-produced BRAD-3 was provided by Sanquin (Amsterdam, The Netherlands). Prior to use, the BRAD-3 sample was affinity purified using protein G Sepharose beads (see **S-1.3**, Supporting Information). Plasma IgG was isolated from human donor plasma by affinity purification also using protein G Sepharose beads (see **S-1.4**, Supporting Information).

The oligosaccharide standards 3'-sialyllactose (Neu5Acα2,3Galβ1,4Glc) sodium salt and 6'-sialyllactose (Neu5Acα2,6Galβ1,4Glc) sodium salt were purchased from Carbosynth (Compton, U.K.) and the 2-aminobenzamide (2-AB) labeled triantennary (A3) glycans were obtained from Ludger (Oxfordshire, U.K.).

### 2.2.3 Preparation and analysis of glycans and glycopeptides

Digestion of the isolated plasma IgGs (approx. 1 mg/mL) was performed by TPCK treated trypsin, added in a 1:10 (enzyme:substrate) ratio to the sample. The samples were shaken for 10 min at 600 rpm, after which the pH was checked and found to be in a range from pH 6 to 10. Subsequent overnight incubation at 37 °C resulted in a tryptic digest of 0.99 mg/mL plasma IgG. For the IgG1 standard, mAb1, mAb2, the bovine IgG and BRAD-3, the proteins were partially denatured prior to digestion by the addition of 20 µL 100 mM FA to 1 µL protein solution, followed by a 15 min incubation at RT. Subsequently, the samples were vacuum dried at 60 °C and dissolved in 50 mM sodium bicarbonate. Trypsin was added in a 1:10 (enzyme:substrate) ratio to the samples and the mixtures were incubated overnight at 37 °C. In this way, tryptic digests were obtained containing 0.23 mg/mL IgG1, 0.25 mg/mL mAb1, 0.19 mg/mL mAb2, 0.25 mg/mL bovine IgG or 0.25 mg/mL BRAD-3. *N*-glycans were released from the IgG samples by PNGase F digestion (see **S-2.1**, Supporting Information)[167] and subjected to linkage-specific derivatization of the sialic acids by ethyl esterification (see **S-2.2**, Supporting Information) [124].

Both glycans and glycopeptides were purified by cotton hydrophilic interaction liquid chromatography (HILIC) solid phase extraction (SPE) (see **S-2.3**, Supporting Information) [168]. Subsequent MALDI-TOF(/TOF)-MS analysis and data analysis were performed as described in **S-3**, **S-4** and **Table S-1**, Supporting Information.

### 2.2.4 Comparison of different nucleophiles for glycopeptide derivatization

Tryptic IgG glycopeptides derived from human plasma were subjected to derivatization with ethanol, methanol or dimethylamine using EDC and HOBt, to find the most suitable reagent for linkage-specific sialic acid modification with minimal reaction variability on the peptide.

Methyl and ethyl esterifications were performed on 1 µL tryptic IgG glycopeptides in 20 µL 250 mM EDC and 250 mM HOBt in the respective alcohol for 1 h at 4 °C and 37 °C, respectively. These are conditions which were reported to result in linkage-specific sialic acid derivatization [124]. After incubation, 20 µL of ACN was added and the derivatized glycopeptides were purified by HILIC-SPE followed by MALDI-TOF-MS analysis.

To study the use of dimethylamine for the derivatization of IgG glycopeptides, EDC, HOBt and dimethylamine were dissolved in DMSO to concentrations of 50, 150 or 250 mM. Further optimization was performed by increasing the HOBt concentration to 350 and 500 mM while keeping both the EDC and the dimethylamine at 250 mM. Combining 1 µL of the IgG digest with 20 µL of the respective reagent mixtures, incubation was performed for 3 h at 60 °C. After addition of 113.3 µL ACN (yielding a concentration of 85%), derivatized glycopeptides were purified from the mixture by HILIC-SPE, followed by MALDI-TOF-MS analysis.

The level of variability in the modifications on the peptide portion was for all methods determined based on the G1F glycoform. For the ethyl esterification and the dimethylamidation the IgG1 signals with different modifications were extracted from the spectra. For the methyl esterification the IgG2 signals were extracted. Averages and standard deviations were calculated for three replicates.

### 2.2.5 Dimethylamidation of samples with different sialic acid linkages

To profile the glycosylation of the IgG1 standard, mAb1, mAb2, bovine IgG and BRAD-3 fragment crystallizable region, 1 µL of their digest was derivatized for 3 h at 60 °C, using 20 µL of the optimal dimethylamidation reagent (250 mM EDC, 500 mM HOBt and 250 mM dimethylamine in DMSO). Samples were purified by cotton HILIC-SPE and measured by MALDI-TOF-MS.

The 6'- and 3'-sialyllactose standards were 2-AB labeled as described in **S-5**, Supporting Information. These and the 2-AB labeled A3 glycan standard were dimethylamidated, followed by MALDI-TOF-MS measurement**.**

## 2.3 Results

Here, we present a method for the derivatization of IgG glycopeptides from both human plasma and biopharmaceutical sources with selective reactivity for different sialic acid linkages, thus allowing IgG subclass- and sialic acid-linkage-specific glycosylation analysis by MALDI-TOF-MS.

### 2.3.1 IgG glycopeptide derivatization conditions

We previously reported sialic acid linkage-specific esterification of released glycans using the carboxylic acid activator EDC and the catalyst HOBt in ethanol (37 °C) and methanol (4 °C) [124]. These conditions had been optimized to provide selective reaction products for α2,3-linked and α2,6-linked sialic acids. Here, these esterification conditions were applied to the derivatization of tryptic glycopeptides derived from human plasma IgG, and the modifications induced in the glycan and peptide moieties were investigated.

All IgG subclasses proved to be recoverable by HILIC-SPE from the 1:1 ethanol/methanol:ACN conditions, and reflectron positive ion mode MALDI-TOF-MS allowed the detection of the native, the ethyl esterified and the methyl esterified glycopeptides as $[M+H]^+$ ions (**Figure 2.1**, for a full listing of peaks detected throughout the experiments after internal calibration see **Table S-2**, Supporting Information). The most intense signals represent core-fucosylated diantennary glycans carrying no galactose (G0F, *m/z* 2634.044 (IgG1 – native)), one galactose (G1F, *m/z* 2796.095), or two galactoses (G2F, *m/z* 2958.150), with other glycan species varying in fucosylation, bisection and sialylation. For the native IgG glycopeptides only a low degree of sialylation was visible (13% of the main peak), as well as metastable signals indicative for sialic acid loss (**Figure 2.1A**). Ethyl and methyl esterification conditions did not show this breakdown, with a concurrent higher degree of sialylation (29% and 31% of the main peak, **Figure 2.1B and 2.1C**).

All IgG subclasses contain three carboxylic acids in their glycopeptide sequence; two on the glutamic acids (E) and one on the C-terminus (IgG1 = EEQYNSTYR, IgG2/3 = EEQFNSTFR, IgG4 = EEQFNSTYR). When comparing the esterified and native conditions, the non-sialylated glycopeptide species show a mass increase corresponding to two times esterification and one loss of water (38.052 Da and 10.021 Da for ethyl and methyl esterification, respectively), likely to be positioned at the carboxylic acids of the peptide portions. The sialylated glycopeptide species (carrying one α2,6-linked sialic acid) show an additional mass increase upon esterification (28.031 Da or 14.016 Da for ethyl and methyl esterification), suggesting derivatization of the sialic acid as well.

Detrimentally, a byproduct was formed under ethyl and methyl esterification conditions for all glycopeptide species (indicating a peptide-specific effect), corresponding to the

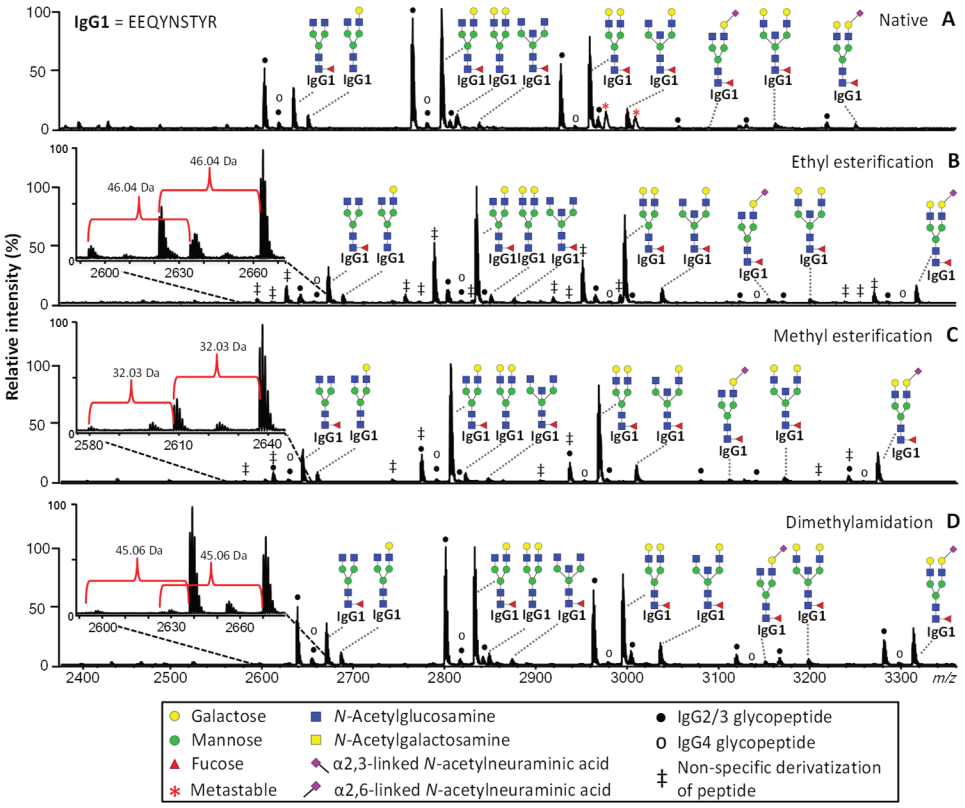


**Figure 2.1 MALDI-TOF-MS spectra of plasma IgG glycopeptides.** (**A**) Without derivatization, (**B**) after ethyl esterification, (**C**) after methyl esterification and (**D**) after dimethylamidation. Derivatization (**B-D**) prevents the formation of metastable signals, and increases the detection of sialylated species. Magnifications of the m/z region corresponding to the G0F-carrying glycopeptides show the variable nucleophilic addition and water loss of the peptide portion after ethyl esterification (-46.042 Da) and methyl esterification (-32.026 Da), with dimethylamidation showing the least side-reactivity (-45.058 Da). Except for the sialic acid linkage, the monosaccharide linkages were not determined. Proposed glycan structures are based on fragmentation and literature [29, 74, 169, 170].

exchange of one esterification for the loss of water (-46.042 Da and -32.026 Da, respectively). When compared to the intensity of the major reaction product, the byproduct showed 10.0% relative abundance (standard deviation (SD) ± 4.2%) and 33.3% relative abundance (SD ± 4.6%) for methyl esterification and ethyl esterification respectively. Unfortunately, the resulting mass difference is equal to the mass difference expected between α2,3- and α2,6-linked sialylation, preventing potential relative quantification of the sialylation isomers. In addition, while methyl esterification shows the

lowest variability, its relative quantification is further complicated by the fact that the mass difference between methylated and lactonized species (32.026 Da) is very similar to the mass difference between the IgG subclasses IgG1 and IgG2/3 (31.990 Da), resulting in multiple isobaric species.

Next to esterification, the IgG glycopeptides were subjected to amidation with ammonia, methylamine and dimethylamine. Method optimization to maximize specificity of the reaction with respect to both glycan and peptide derivatization resulted in final conditions of 250 mM dimethylamine, 250 mM EDC and 500 mM HOBt in DMSO with incubation for 3 h at 60 °C (**Figures S-1** to **S-3**, Supporting Information). Performing reflectron positive mode MALDI-TOF-MS on the dimethylamidated samples showed stabilization of the sialylation (42% of the main peak) and the absence of metastable signals (**Figure 2.1D**). For the peptide portion, two times dimethylamidation and one time water loss was detected, with the α2,6-linked sialic acids showing dimethylamidation as well. Importantly, the variability seen in the esterified glycopeptide samples is present in only minor amounts for the dimethylamidation condition (1.1% relative abundance; SD ± 0.2%).

### 2.3.2 Sialic acid linkage-specificity of dimethylamidation

To assess the selectivity of the dimethylamidation reaction for differently linked sialic acids, two sialyllactose standards with different sialic acid linkages and glycopeptides from four IgG standards were subjected to the dimethylamidation conditions.

Commercially available plasma IgG1, carrying mainly α2,6-linked sialic acids, showed full dimethylamidation of the sialylated species (*m/z* 3312.377, **Figure 2.2A**). MAb1, which is known to contain exclusively α2,3-linked sialic acids [171], showed primarily lactone formation of its sialic acids (*m/z* 3267.320, **Figure 2.2B**), just like the more highly α2,3-sialylated BRAD-3 (*m/z* 3251.326, **Figure S-4**, Supporting Information). In addition, BRAD-3 showed 4.0% dimethylamidation of the sialic acids (+45.058 Da, for observed side reactions see **Table S-3**, Supporting Information). Due to traces of ammonia in the reaction mixture of mAb1, also 10.4% (SD ± 3.2% for 36 samples) amidation with ammonia was seen for the α2,3-linked sialic acids (*m/z* 3284.347). This contaminant most likely comes from the mAb1 sample itself, as BRAD-3 did not show this particular side-reaction. In contrast, the amidation with ammonia was not observed to compete with the lactamization or dimethylamidation on the peptide backbone. The main products after dimethylamidation showed a mass difference of 45.058 Da between the differently linked sialic acids (**Figure 2.3**). This could also be confirmed with sialyllactose standards, showing 99.5% (SD ± 0.1%) dimethylamidation on 6'-sialyllactose and 92.3% (SD ± 0.5%) lactone formation on 3'-sialyllactose (**Figure S-5**, Supporting Information). MAb2, derived from mAb1 by modifying its glycosylation in vitro using galactosyltransferases and sialyltransferases, contains glycan structures that are high in

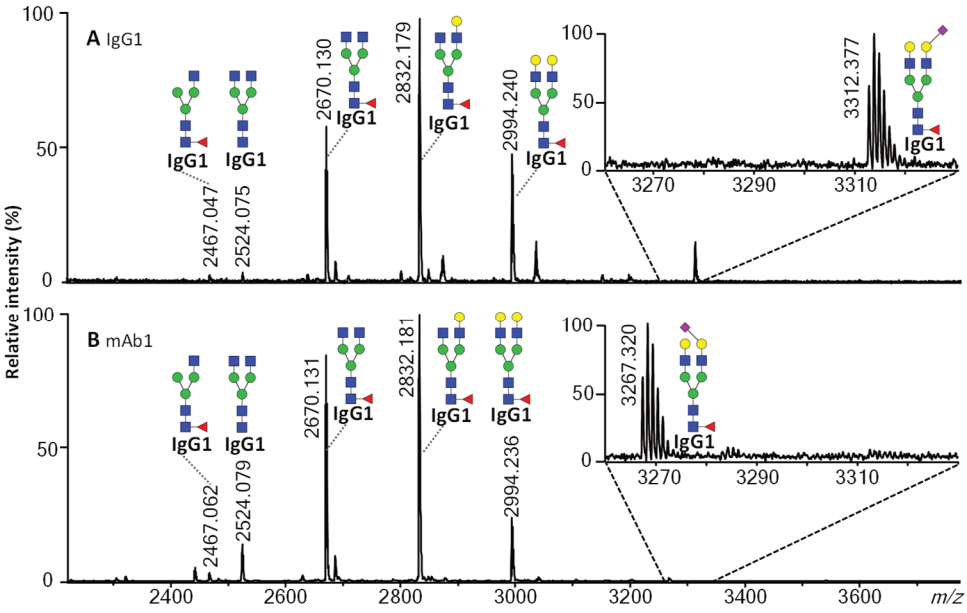


**Figure 2.2 MALDI-TOF-MS spectra of the glycopeptide profiles of (A) the IgG1 standard and (B) mAb1 after dimethylamidation.** The magnifications focus on the sialylated glycopeptides of the antibodies. Plasma IgG1 mainly carries α2,6-linked sialic acids, which form a dimethylamide during derivatization (+27.047 Da), while mAb1 mainly carries α2,3-linked sialic acids, which yields water loss during derivatization (-18.011 Da). After linkage-specific derivatization of the sialic acids, the mass difference between α2,3- and α2,6-linked forms is 45.058 Da. For α2,3-linked sialic acids side products amidated with ammonia (-0.984 Da) were found.

galactosylation and contain both α2,3- and α2,6-linked sialic acids. Glycosylation of tryptic mAb2 glycopeptides was analyzed by dimethylamidation using the newly established protocol. The MALDI-TOF-MS profile obtained (**Figure 2.4B**) was compared to a profile resulting from the established method of ethyl esterification after glycan release (**Figure 2.4A**). The profiles were highly comparable for the 19 glycoforms detected (**Figure 2.4C**). The most abundant glycan (G2FS – α2,6-linked) showed in the released glycan assay an average relative area of 60.0% (SD ± 0.2%) and in the glycopeptide assay an average relative area of 59.9% (SD ± 0.9%) across triplicate measurements. The difference across the other species was found to be at most 2.7% of the total relative area (G2FS2). In addition, the obtained glycosylation profile of mAb1 is in very good agreement with the previously published HILIC-UPLC data for released glycans of this same antibody (**Figure S-6**, Supporting Information) [171], proving that possible side reactions do not interfere with the profiling of the glycopeptides.

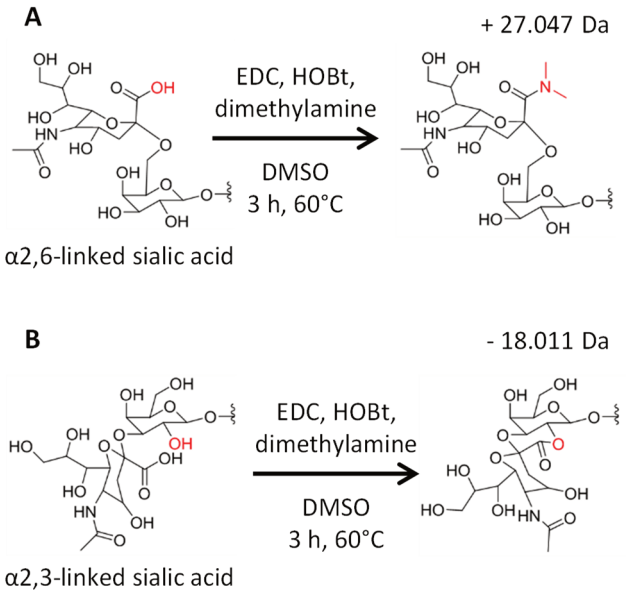


**Figure 2.3 Reaction scheme for the derivatization of *N*-acetylneuraminic acid that is either (A) α2,6-linked or (B) α2,3-linked to the subterminal galactose.** Under the same conditions, α2,6-linked sialic acids form a dimethylamide, whereas α2,3-linked sialic acids react with the neighboring galactose to form a lactone.

The dimethylamine derivatization was additionally performed on a 2-AB labeled triantennary glycan standard containing glycans with a variety of sialic acid linkages. The relative ratio of these species had previously been determined by HILIC-UPLC with exoglycosidase digestion, and shown to correspond to MALDI-TOF-MS analysis of the same sample after ethyl esterification [172]. Comparing esterification with the here described dimethylamidation again shows highly similar ratios and indicates equal response factors for the differentially linked sialic acids after derivatization (**Figure S-7**, Supporting Information). In addition to the stabilization of *N*-acetylneuraminic acids in a linkage specific way, the derivatization method was found to be applicable to IgG glycopeptides containing *N*-glycolylneuraminic acids derived from bovine IgG (**Figures S-8** and **S-9**, Supporting Information).

### 2.3.3 Repeatability

To demonstrate the robustness of the dimethylamidation protocol, 36 aliquots of the IgG1 standard and mAb1 samples were prepared. On three successive days, 12 aliquots of each antibody were subjected to tryptic digestion, dimethylamidation for 3 h at 60 °C, HILIC-SPE and MALDI-TOF-MS analysis.

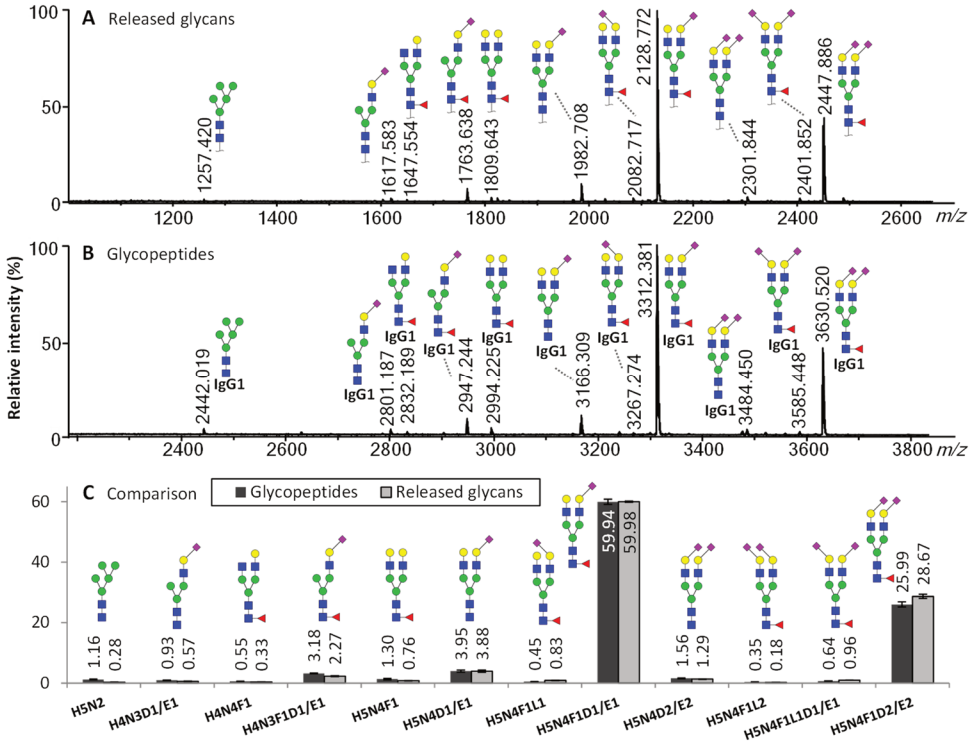


**Figure 2.4 MALDI-TOF-MS analysis of mAb2 glycosylation.** (**A**) Profile of the released glycans after ethyl esterification. (**B**) Profile of the tryptic glycopeptides after dimethylamidation. (**C**) Average relative intensities for the major glycoforms over three samples per profiling method, with error bars for replicate standard deviation. Abbreviations used are hexose (H), *N*-acetylhexosamine (N), fucose (F), dimethylamidated *N*-acetylneuraminic acid with an α2,6-linkage (D), ethyl esterified *N*-acetylneuraminic acid with an α2,6-linkage (E) and lactonized *N*-acetylneuraminic acid with an α2,3-linkage (L).

The established method with the optimized derivatization procedure showed very good precision, both intra-day and inter-day (**Figure S-10**, Supporting Information). The average relative area of the highest peak for the IgG1 standard (G1F) was 35.2% (SD ± 0.8%) across all samples, resulting in an average coefficient of variation (CV) of 2.2%. For mAb1 the relative area of the G1F peak was 42.9% (SD ± 1.1%) with an average CV of 2.5%. Besides the highest signals in the spectra, also the (low abundant) sialylated glycopeptides showed an acceptable CV between 4% and 18%, implying that both α2,3- and α2,6-linked sialic acids are stabilized and detected in a repeatable way.

### 2.3.4 Fragmentation

Both the native form of the IgG1 glycopeptide, carrying G2FS (α2,6-linked; *m/z* 3249.248), as well as the dimethylamidated form of this analyte (*m/z* 3312.384) were subjected to positive ion mode MALDI-TOF/TOF-MS/MS fragmentation to show the effects of sialic acid stabilization in MS/MS. In addition, a derivatized non-sialylated glycopeptide (IgG1 carrying G0F; *m/z* 2670.134) was fragmented to determine the modifications on the peptide portion of the conjugate.

Fragmentation of native glycopeptides predominantly showed the loss of the labile sialic acid (-291.1 Da) (**Figure 2.5A**). Other minor fragments seen were the peptide portion containing a cross-ring fragment of the Asn-linked *N*-acetylglucosamine (*m/z* 1271.9) as well as a fragment carrying the innermost *N*-acetylglucosamine with the core fucose (*m/z* 1538.0).

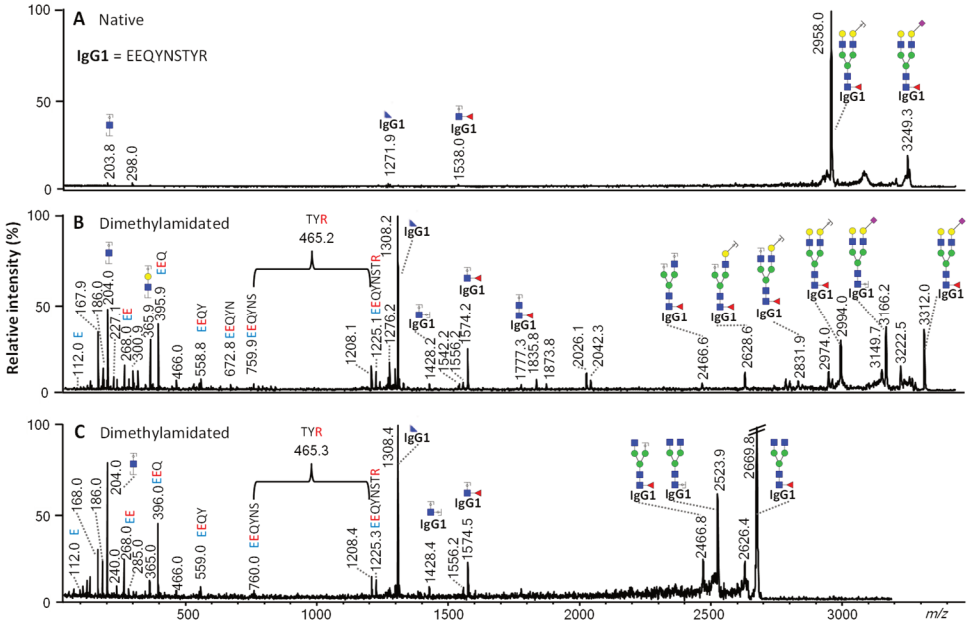


**Figure 2.5 MALDI-TOF/TOF-MS/MS spectra of the IgG1 G2FS glycopeptide (A) without derivatization and (B) after dimethylamidation.** Whereas the unmodified glycopeptide mainly shows fragmentation of the sialic acid, the derivatized variant shows fragmentation of both the glycan and the peptide moiety. (**C**) Shows the fragmentation of a non-sialylated IgG1 glycopeptide (G0F) after dimethylamidation, demonstrating the reactions occurring on the peptide portion of the conjugate. Blue: Loss of water. Red: Dimethylamide formation.

After derivatization, the fragmentation spectra were considerably more informative with respect to both glycan and peptide moiety (**Figure 2.5B**), showing not only loss of the dimethylamidated sialic acid (-318.1 Da), but also various other losses indicative for antenna composition. Fragments originating from the peptide portions of both G2FS and G0F (**Figure 2.5B and 2.5C**) show water loss and dimethylamidation on the two N-terminal glutamic acids (*m/z* 268.0 $[M + H]^+$). This likely reflects pyroglutamic acid formation of the N-terminal glutamic acid and dimethylamidation of the intra-chain glutamic acid. Dimethylamidation at the three most C-terminal amino acids was observed (a loss of 465.2 Da from *m/z* 1225.1 to *m/z* 759.9) pointing to the modification of the C-terminal carboxylic acid. Fragmentation of the esterified glycopeptides revealed that the intra-chain glutamic acid is involved in the appearance of the side-reactivity under esterifying conditions (**Figure S-11**, Supporting Information). Lactone formation (-18.01 Da) of α2,3-linked sialic acids upon derivatization was further established by the fragmentation pattern of the triantennary glycan with two α2,3-linked and one α2,6-linked sialic acid from the A3-2-AB glycan standard (**Figure S-12**, Supporting Information).

## 2.4 Discussion

Derivatization of sialylated glycopeptides for MALDI-TOF(/TOF)-MS analysis has previously been described [128, 166], but the protocol presented here is the first that includes the linkage-specific modification of sialic acids. For free glycans, the possibility to use the difference in reactivity between α2,3- and α2,6-linked sialic acids for the specific modification of both isomers has been reported previously [123, 124, 162]. However, the set-up of a protocol for glycopeptides adds complexity due to the presence of peptide moiety carboxylic acids. These acids could participate in a wide range of reactions depending on their local environment. Potential side reactions would include either internal or external esterification, amidation or thioester formation. Furthermore, the behavior of the modified glycoconjugates is likely to be altered with respect to solubility, HILIC retention and ionization. Consequently, the methyl and ethyl esterification of tryptic IgG glycopeptides could not readily be controlled, and heterogeneous reaction products were observed for the peptide portion of the conjugates. Fragmentation shows the N-terminal glutamic acid to mainly lactamize into pyroglutamic acid while the C-terminus forms an alkyl ester. The observed reaction variability seems to be caused by the intra-chain glutamic acid, possibly forming a lactone with a nearby amino acid (e.g. with the serine or threonine hydroxyl group) as a side reaction to the intermolecular ester formation. While some side-reactivity can be allowed, the mass shifts induced by the peptide variability overlap with the mass difference caused by the peptide portion heterogeneity of IgG subclasses, and are equal to the mass shifts observed for differentially linked sialic acids, consequentially prohibiting relative quantification.

As we expected EDC and HOBt to lead to linkage-specific sialic acid modification when presented with a suitably sterically hindered nucleophile [124], we decided to investigate the nucleophile selection rather than, for example, perform an exploration of carboxylic acid activators. Ammonia, methylamine and dimethylamine were all found to stabilize the sialylated glycopeptides for MALDI-TOF(/TOF)-MS measurements, but as expected, the use of the smaller amines caused a decrease in selectivity for the sialic acid linkage (data not shown). The more slowly reacting dimethylamine allowed high sialic acid linkage-specificity of the reaction, and it proved specific for the different peptide carboxylic acids as well. In addition, the use of a secondary amine will also prevent possible cross-linking of several carboxylic acid moieties which may be observed for primary amines or ammonia [173]. The resulting amidation reaction conditions (3 h at 60 °C) are harsher than those previously reported for the ethyl esterification [124], but are still within the scope of published glycan derivatization methodology [123, 174, 175]. For the glycopeptides tested here, no signs of either peptide or glycan degradation were observed.

The reaction with dimethylamine was shown to be highly suitable for the glycosylation profiling of therapeutic monoclonal antibodies as demonstrated for mAb1 and mAb2, resulting in relative ratios comparable to previously established methods [124, 171]. The resolution of the method was sufficient to analyze complex IgG samples with various sialic acid linkages, different subclass peptide sequences, and both *N*-glycolyl- and *N*-acetylneuraminic acids in the structure. With a potential sample throughput of 384 samples a day, the method is rapid compared to existing sialylated glycopeptide profiling methods like LC-MS and CE-MS.

While optimized for IgG glycopeptides carrying α2,3- or α2,6-linked *N*-acetyl- and *N*-glycolylneuraminic acids, the effect of the reaction on other sialylation variants is still unknown, examples of this being α2,8-linkage and *O*-acetylation. In addition, it will be interesting to study the effect of the method on the glycopeptides originating from proteins other than IgG. However, due to the great variability in the amino acid sequence of different glycopeptides, the protocol is expected to require optimization to ensure uniformity in the reactions occurring on the peptide portion of the conjugates.

In conclusion, we presented a method for the stabilization of sialylated glycopeptides for MALDI-TOF(/TOF)-MS analysis, which can be used for the high-throughput linkage-specific analysis of sialylated glycopeptides derived from IgG in a subclass- and site-specific manner. Derivatization induces a mass difference between α2,3- and α2,6-linked sialic acids, while peptide modifications are uniform, enabling separation between differently linked sialic acids in MS. Furthermore, highly informative MS/MS spectra of sialylated glycopeptides could be obtained. The method is fast, has excellent intra- and inter-day repeatability and makes use of relatively inexpensive chemicals. A considerable

applicability is expected for the future glycosylation profiling of therapeutic antibodies, as well as for the IgG glycosylation profiling of large clinical cohorts.

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## Supporting Information available

Additional information is available as stated in the text. This information is available free of charge via https://pubs.acs.org/doi/10.1021/acs.analchem.5b02426.

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