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Novel data analysis tool for semiquantitative LC-MS-MS^2 profiling of N-glycans

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Abstract Despite recent technical advances in glycan analysis, the rapidly growing field of glycomics still lacks methods that are high throughput and robust, and yet allow detailed and reliable identification of different glycans. LC-MS-MS^2 methods have a large potential for glycan analysis as they enable separation and identification of different glycans, including structural isomers. The major drawback is the complexity of the data with different charge states and adduct combinations. In practice, manual data analysis, still largely used for MALDI-TOF data, is no more achievable for LC-MS-MS^2 data. To solve the problem, we developed a glycan analysis software *GlycanID* for the analysis of LC-MS-MS^2 data to identify and profile glycan compositions in combination with existing proteomic software. IgG was used as an example of an individual glycoprotein and extracted cell surface proteins of human fibroblasts as a more complex sample to demonstrate the power of the novel data analysis approach. N-glycans were isolated from the samples and analyzed as permethylated sugar alditols by LC-MS-MS^2, permitting semiquantitative glycan profiling. The data analysis consisted of five steps: 1) extraction of LC-MS features and MS^2 spectra, 2) mapping potential glycans based on feature distribution, 3) matching the feature masses with a glycan composition database and *de novo* generated compositions, 4) scoring MS^2 spectra with theoretical glycan fragments, and 5) composing the glycan profile for the identified glycan compositions. The resulting N-glycan profile of IgG revealed 28 glycan compositions and was in good correlation with the published IgG profile. More than 50 glycan compositions were reliably identified from the cell surface N-glycan profile of human fibroblasts. Use of the *GlycanID* software made relatively rapid analysis of complex glycan LC-MS-MS^2 data feasible. The results demonstrate that the complexity of glycan LC-MS-MS^2 data can be used as an asset to increase the reliability of the identifications.

Keywords Glycomics · Mass spectrometry · Bioinformatics · Immunoglobulin · Fibroblast

Abbreviations

G	*N*-glycolylneuraminic acid Neu5Gc
ESI	Electrospray ionization
F	Deoxyhexose (fucose)
H	Hexose Hex
ISD	In-source decay
IVIG	Intravenous immunoglobulin
LC	Liquid chromatography
LTQ	Linear trap quadrupole
MALDI	Matrix-assisted laser desorption ionization
MS	Mass Spectrometry
MS^2	Tandem Mass Spectrometry
N	*N*-acetylhexosamine HexNAc
NHDF	Normal human dermal fibroblasts
RP	Reversed-phase
RT	Retention time
S	*N*-acetylneuraminic acid Neu5Ac (sialic acid)
TOF	Time-of-flight

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Introduction

There is an increasing need to characterize and quantify glycan moieties of glycoproteins. The changes in glycan compositions and quantity are indicative of the biological function of glycan carrying cells or molecules, one striking example being antibody glycosylation. There is only one conserved glycosylation site in human IgG (Asn297). Although IgG N-glycan represents only small part of the total mass of the molecule, the specific glycoforms of IgG are essential to immune effector functions of antibodies [1]. Recently, engineering of IgG glycoforms of therapeutic monoclonal antibodies has become important to provide pre-selected N-glycosylation and therefore maximum efficacy for a given disease indication. Further, the anti-inflammatory activity of intravenous IgG (IVIG) is dependent on sialylation of the N-linked glycan of IgG Fc fragment [2]. Another easily interpreted biological function for glycosylation is the cellular interaction, *e.g.* in adhesive functions. Therefore the determination of cell surface glycan profiles has become more and more evident in studying cellular functions, especially for cells aimed at therapy [3, 4].

Traditionally, mass spectrometric (MS) glycan analysis has mainly been performed by one-dimensional matrix assisted laser desorption ionization (MALDI) – time-of-flight (TOF) analysis [5, 6], whereas in proteomics the use of liquid chromatography (LC) coupled to electrospray ionization (ESI)-MS has increased rapidly in recent years in order to identify proteins in complex mixtures. For glycans there are many good reasons to favour MALDI, including simpler one-dimensional spectra, established wetlab procedures, existing software *etc.* The benefit of the LC is on the other hand in additional glycan separation, which permits for example the isomeric differentiation of glycans [7, 8]. Tandem mass spectrometric (MS^2) fragmentation analysis is also easier to perform on an ESI-MS instrument, although it is feasible today also on some MALDI instruments. The use of LC-MS-MS^2 has been limited both by the complexity of the spectra, namely in the form of multiple different charge states and adducts, and by expensive and relatively difficult-to-use instrumentation. But in particular, the major drawback is the lack of suitable software to ease the LC-MS-MS^2 data analysis.

The aim of glycan identification is to find the set of glycan compositions that explains the data best, and to calculate the total intensities, *i.e.* profile, for the identified glycans. Several software tools to analyze glycan mass spectra are available, including the free GlycoWorkbench [9], Glyco-Peakfinder [10], GlycoMod [11], Manatee [12], and the proprietary SimGlycan (www.premierbiosoft.com). Further, software called SysBioWare [13] is aimed for glycan specific LC-MS raw data processing and glycan mass matching. All the listed software have comprehensive features for glycan mass matching, two of those (GlycoWorkbench and SimGlycan) also support matching glycan structures with MS^2 spectra. However, they have been developed for the analysis of one spectrum at a time and their automation for a larger set of data acquired by LC-MS-MS^2 analysis, containing both LC-MS (features) and MS^2 spectra, either is not possible or their capability is limited.

For data analysis of peptide LC-MS experiment, plenty of software exist both as open source (for example msInspect [14] and OpenMS [15]) and as proprietary ones (for example Progenesis LC-MS by Nonlinear Dynamics Ltd (www.nonlinear.com) and DeCyder by GE Healthcare Ltd (www.gelifesciences.com)). Peptide LC-MS analysis differs slightly from the corresponding glycan analysis, where there are several charge carriers, not just proton adducts as in peptides. But there are also many similar attributes in the LC-MS data analysis of these two analytes, peptides and glycans, including feature detection, alignment and feature comparison between different samples.

The comparative and comprehensive glycan analytical tool should be able to (a) detect and quantify as many different glycan types as possible and (b) separate isomeric and isobaric glycan structures. To improve resolution of complex glycomic samples, we chose in this study to use a two-dimensional LC-MS-MS^2 method which is a combination of chromatographic and mass based separation. The application of glycan permethylation permitted semiquantitative glycan profiling as it is known to reduce differences in ionization efficiency between different glycans [16]. To simplify the interpretation of the data, new software tool *GlycanID* was developed (presented in: [17]) and combined with the existing proteomic software, which can be utilized for data pre-processing. The *GlycanID* glycomic software consists of a function library developed with R (www.r-project.org) and Perl (www.perl.org) programming languages and is aiming at automated, batch process type data analysis and methods that can be easily modified in case new requirements arise. The identification of glycan compositions was set to be sufficient, and if more detailed results are needed, other software like GlycoWorkbench can be used to refine the results for few selected MS^2 spectra.

The key ideas of our method are 1) mapping the most relevant (high abundance) feature data, 2) multiple search cycles of MS (feature) and MS^2 matches with different adducts and 3) grouping the resulting matches so that the most probable set of identified glycans can be evaluated and the profile can be constructed. In this study we investigated the glycosylation of purified IgG as well as glycosylation of the cell surface enriched protein pool of human fibroblasts to demonstrate the power of the novel *GlycanID* data analysis approach. We used in-house developed reversed-phase LC separation of permethylated glycans prior the MS-MS^2 analyses (manuscript in preparation). Separation of

glycans prior to mass analysis and the superior accuracy of LC-MS yielded high amounts of information. Combined with semiquantitative glycan profiling this enabled the identification of glycan compositions from a complex mixture. The complexity of the data was utilized to increase the reliability of the method as the glycan identifications are based on several data points, improving the resolving power of the method.

Materials and methods

Sample information

Intravenous immunoglobulin G (IVIG) Octagam was obtained from Octapharma (Stockholm, Sweden). Normal human dermal fibroblasts (NHDF) from adult donor (cryopreserved, PromoCell, Heidelberg, Germany) were cultured in Fibroblast Growth Medium 2 with Supplement Mix purchased from PromoCell. For cell surface glycan analysis, cells were detached by trypsinization (TrypLE Express, Gibco, Gaithersburg, MD, USA) and washed with PBS. The cell surface protein fraction was enriched using biotinylation of intact cells and further captured using streptavidin coupled magnetic beads, as previously described [18].

Tryptic digestion

Both IgG (5 μg) and enriched cell surface protein fraction of human fibroblasts (from 2.5×10^6 cells) were digested with immobilized TPCK trypsin (Pierce, Rockford, IL) at 37 °C overnight with rapid shaking. The reaction was stopped by adding the reaction mixture on the top of PD MiniTrap G-10 column (GE Healthcare, Uppsala, Sweden) equilibrated with 20 mM NH_4HCO_3. The glycopeptide fraction was then eluted and collected according to the manufacturer's instructions.

N-glycan purification and derivatization

Vacuum dried glycopeptides were dissolved in 20 μl of 25 mM NH_4HCO_3 and 5 μl (2.5 U) of PNGase F (Sigma Aldrich) was added. The reaction mixture was incubated overnight at +37 °C. After drying under vacuum and dissolving with 5 % acetic acid the peptide material was removed from the reaction mixture by using C18 ZipTip (Millipore, Billerica, MA, USA). The released N-glycans were reduced by using $NaBH_4$ in 10 mM NaOH. The reduced N-glycan alditols were desalted by using graphitized carbon pipette tips (TopTip, Glygen, Columbia, ND) and permethylated by a solid-phase spin-column technique essentially as described [19]. The reaction mixture was then dried under vacuum to remove acetonitrile and permethylated N-glycan alditols were purified thereafter with C18 ZipTip and dried under vacuum.

Mass spectrometry

Reduced and permethylated N-glycan alditols were analysed by LC-MS-MS^2. Glycans were loaded to a reversed-phase precolumn (NanoEase Atlantis dC18, 180 μm× 23.5 mm,Waters) with 90 % of solvent A (0.2 mM NaOH in 0.1 % formic acid) and 10 % of solvent B (90 % acetonitrile, 10 % isopropanol and 0.1 % formic acid). Glycans were separated in reversed-phase analytical column (PepMap 100, 75 μm×150 mm, Dionex Corporation) with linear gradient (40–90 %) of solvent B in 30 min. Ultimate 3,000 liquid chromatography instrument (Dionex Corporation) was operated in nano scale with flow rate of 0.3 μl/min. Eluted glycans were introduced to LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific Inc.) *via* ESI Chip interface (Advion BioSciences Inc.) in positive-ion mode.

The mass spectrometer was calibrated with Thermo Fisher Scientific standard LTQ calibration solution consisting of caffeine, MRFA tetrapeptide and Ultramark 1,621. Full scan for eluting glycans was acquired in mass range of 300–2,000 m/z on Orbitrap-detector with 60,000 resolution (Full width at half maximum, FWHM) at 400 m/z. Based on full scan, top three eluting glycans were fragmented in data-dependent manner using both CID and HCD method. MS^2 scans were acquired on Orbitrap-detector with 7,500 resolution (FWHM) at 400 m/z. Precursors, whose charge state couldn't be determined were discarded from MS^2 analysis. Both full scan and MS^2 scans consisted of one microscan and they were acquired as profile data.

Data analysis in feature extraction

Progenesis LC-MS software (Version 2.6, Nonlinear Dynamics Ltd.) was used for LC-MS differential expression analysis and to extract MS level data (features). MS^2 data was extracted with Mascot Distiller (Matrix Science Ltd., version 2.3.1). A novel in-house developed software *GlycanID* was used to derive glycan compositions from MS and MS^2 data.

Results

GlycanID as a novel tool in glycomics

We introduce a novel in-house developed software *GlycanID* in order to resolve complex LC-MS-MS^2 data on glycans, which in the present study were permethylated N-glycans with a relatively predictable fragmentation pattern [20]. Because permethylation renders oligosaccharides nearly

chemically equivalent in the mass spectrometer, the method is semiquantitative [16]. Permethylation also enables simultaneous analysis and quantification of neutral and sialylated glycans and stabilizes the relatively labile sialic acid residues.

The first step in the GlycanID workflow was to mark the feature and MS^2 data that most likely contained a glycan signal. A feature with a charge state z was marked to match a *pattern*, if there were, within a small RT range ($dRT < 0.2\ min$), z lower intensity and lower mass features separated by the mass difference $(m(sodium)\text{-}m(hydrogen))/z$, where *m(sodium)* is the mass of sodium and *m(hydrogen)* the mass of hydrogen. MS^2 spectra, which contained peaks separated by one or two monosaccharide masses were classified as a potential glycan spectra.

Feature mass matching was then carried out against a glycan composition database and *de novo* generated compositions. Two datasets were used as a glycan database: 1) an in house generated list of compositions including typical contaminants and 2) compositions extracted from GlycomeDB [21]. The combined databases had 2,209 unique compositions. The mass tolerance was set to 7 ppm and the accepted number of residues for the *de novo* search was H:3–12, N:2–12, S:0–5, F:0–5 and G:0–5 (for simplicity single letter code is used, H:Hex, N:HexNAc, S:Neu5Ac, F: Deoxyhexose and G:Neu5Gc). The matching was done several times with different adducts and modifications. At the first round the matching was done against fully permethylated and reduced glycans with sodium adducts only. The following search rounds were done against the matched glycans found at the first round. The search included matching the glycans with hydrogen and sodium adduct combinations, as well as with sodium formate (HCOONa) and with non-reduced glycans. In addition, search of partially permethylated (one missing methyl group) glycans was done, but they were not searched against already found glycans but only against the *de novo* glycans. The reason is that they may result from not only incomplete permethylation reaction, but also from in source decay.

The assumption behind the feature search was that each glycan appears at least once as a fully permethylated and reduced sodium adduct. The other search rounds do not increase the number of identified glycans, but potentially reduce the number of false identifications. The other rounds are also needed for the glycan profile.

The further MS^2 matching was divided into two parts; (a) mass matching with the measured precursors exactly the same way as was done with features and (b) scoring the theoretical fragment spectra with the measured MS^2 spectra. The theoretical spectrum was composed using all monosaccharide combinations that can be derived from a given composition. The result is a spectrum, which is a combination of glycosidic fragments of all structures having the same composition. For example the fragments of the permethylated glycan composition H5N4 are given by,

$$F_{ijkc} = i \cdot m(H) + j \cdot m(N) - (k-1) \cdot m(H_2O) + (2-c) \cdot m(P), \quad (1)$$

where $i = 0..5,\ j = 0..4, 0 < i + j < 9, k = 0..c,\ c = 1..Cmax,$ and $Cmax$ is the largest accepted number of glycosidic cleavages. Masses *m(H)* and *m(N)* are the permethylated residual masses of Hex and HexNAc and $m(H_2O)$ the mass of water and *m(P)* the added mass of methylation (14.01565). When the fragments of the theoretical spectrum were matched with a measured spectrum, the number of peaks in the latter was limited to 100 highest intensity peaks, mass tolerance was set to 15 ppm and max 3 glycosidic cleavages were allowed. The composition match was given a statistical score defined by,

$$S = -(log_{10}(P_H) + log_{10}(P_I)), \quad (2)$$

where P_H is the probability that a random set of fragments would have as many or more shared peaks with the measured spectrum as the ranked composition [22, 23] and P_I is the probability that by randomly selecting the observed number of shared peaks the same or higher amount of intensity could be covered.

The results from MS^2 identification were transformed to the MS features if the mass and retention time differences were less than the given tolerances. The role of MS^2 scoring was to give support for selection of the most probable glycan composition in cases where several glycan compositions matched to a same feature. It cannot be taken as an absolute measure for an existence of a single glycan.

At the last step, the identified glycan compositions were selected from the matched ones and the final glycan profile was calculated. At first, the matching compositions with different adducts were organized so that the compositions, which matched to a same set (or a subset) of features were put into a same group. For each composition the sum of MS^2 scores were calculated from all the matching features with MS^2 spectra. For compositions having S or G residues it was required that the matched MS^2 spectra contained at least one of the given low mass marker oxonium ions (S, SH, SHN or G, GH, GHN). If not, the MS^2 score of that spectrum was not included in the score sum. The identified glycan compositions were selected from the grouped compositions. Compositions, which matched to all the group features, had largest score sum, and had at least one feature with the pattern were selected automatically. Other groups were manually verified and if a glycan composition had a good MS^2 match and there were no contradictions with the other group members the glycan was accepted. In

some cases the identification was also verified be checking the raw data. When the list of identified compositions was available the glycan profile was generated by summing the intensities of matching features for each glycan. Special attention was given to partial permethylation (one methyl group missing). A glycan with a mass matching to partial permethylation was interpreted to be a result of in-source decay (ISD) of a larger (mother) glycan, if both glycans had the same retention time (dRT <0.2 min) and the glycan compositions were such that the fragmentation pattern was plausible. The intensities of glycan fragments were added to the total intensity of the mother glycan. In the case of several possible mother glycans, the intensity was divided to the candidates with the same intensity ratio as the mother glycans had at the same retention time. If a glycan with a partial permethylation was not classified to be a result of ISD and had a retention time with a smaller value than the time of the fully permethylated glycan, the intensity was added to the total intensity of the corresponding glycan.

N-glycan profile of IgG

The N-glycans of therapeutically administered intravenous immunoglobulin (Octagam, OctaPharma, Stockholm, Sweden) were released enzymatically and further permethylated in order to increase the sensitivity and predictability of the LC-MS-MS^2 analyses.

The extracted data on permethylated IgG N-glycans consisted of 549 features and 496 MS^2 precursors within the chosen RT range (13–24 min). By mass matching alone there were 107 different compositions based on feature data and 62 on MS^2 precursor data. Some multiplicity was caused by the mass equality of GF and SH residue combinations. Without the residue G there were 63 unique compositions for features and 38 for MS^2 precursors.

By analyzing the glycan groups, a total of 28 compositions were identified (Table 1 and Fig. 1), 23 suggested by the workflow, and additional 5 evaluated by manual verification. The data contained also some polymer contaminants, (H5, H6 *etc.*) which are not included in the result table. The manually verified compositions included glycans without a pattern but with a good MS^2 match. The data also contained some additional low intensity features with a mass matching to some glycan compositions, but without additional evidence. These 'gray area' glycans were not added to the result, but some of those may be real, especially compositions S1H5N5 and S2H5N5, which have been identified in earlier studies [24]. There was no evidence of multiple retention times for any identified glycan, except non-reduced and partially permethylated forms.

For all the measured features the coverage of the identified glycans was 60 % by the number and 91 % by the total intensity. For the features having pattern 49 (91 %) were identified, and from the 5 features not identified, 4 were classified as noise (matched the side lobes of the highest intensity peaks) and one low intensity feature was left unidentified.

Table 1 Identified IVIG N-glycan compositions. The column labels are: *intensity* is the relative amount (%) of the matched intensity compared to the measured total intensity, *features* is the number of matching features explained by the suggested composition, *patterns* is the number of features matching to the marked pattern

composition	intensity	features	patterns
H4N4F1	20.71	28	3
S1H5N4F1	16.06	38	4
H5N4F1	15.66	34	4
H3N4F1	9.74	17	3
H4N5F1	6.43	19	2
S1H4N4F1	3.75	20	4
H3N5F1	3.70	8	1
H5N5F1	2.32	16	3
S2H5N4F1	2.09	12	2
H5N4	1.94	17	2
S1H5N5F1	1.89	15	2
S2H5N5F1	1.86	21	3
H4N4	1.29	10	1
S1H5N4	0.64	13	1
S2H5N4	0.62	7	1
S1H4N5F1	0.50	9	2
H4N5	0.27	7	2
H4N3F1	0.23	4	1
H3N4	0.22	5	1
H3N3F1	0.22	4	1
H5N5	0.15	3	1
S1H4N3F1	0.11	4	0
H3N5	0.08	4	1
H9N2	0.06	5	1
H8N2	0.04	2	0
S2H6N5F1	0.03	3	0
S1H6N5F1	0.02	2	0
H2N3F1	0.01	1	0
other	9.34	221	5

The profile of the identified glycan compositions is shown at Fig. 2. The total abundance of higher intensity glycans can be divided into two approximately equal size fractions. About half of the total intensity was from fully permethylated and reduced glycans with only sodium adducts, while the other half was divided between glycans with sodium and hydrogen adduct combinations, sodium formate, partially permethylated glycans, non-reduced glycans, and glycans arising from in-source decay. For low intensity glycans the second fraction was considerably smaller, and for the lowest intensity glycan totally absent (Fig. 3). It is reasonable to assume that the lack of the

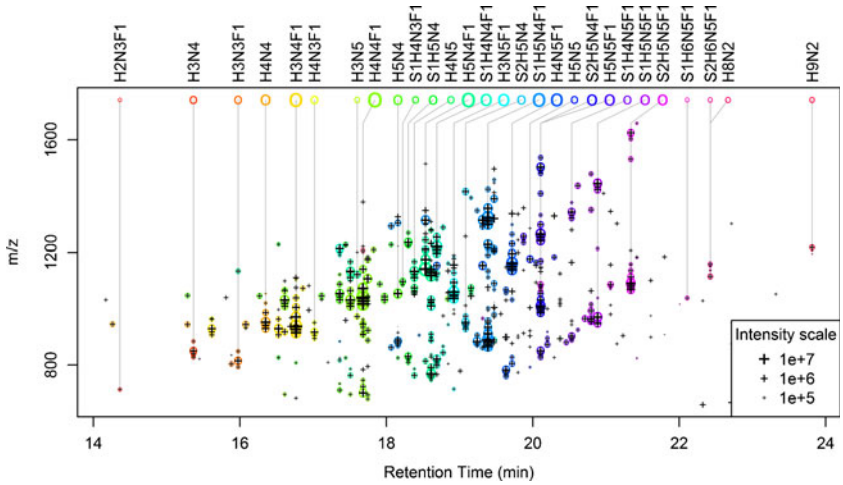

Fig. 1 Map of the IVIG features with identified N-glycan compositions. Glycan labels show the positions of the highest intensity features

second fraction is at least partly due to a detection threshold. For some features that was indeed the case: by looking at the raw data there were few peaks at the expected locations, but the quality was not good enough for the missing features to have been constructed. If that is the case, it causes underestimation of the low intensity glycans relative to the high intensity ones.

The behavior of the glycan variants was found to be rather complex (Fig. 4). Glycans with sodium formate adducts were straightforward, as the retention time was not altered considerably. That was not the case for the non-reduced glycans and for glycans with partial permethylation, which were eluting before the corresponding fully permethylated and reduced glycan. Non-reduced glycans elute in two fractions because of the two different anomeric forms at the reducing end. Also in some cases glycans with partial permethylation were found to elute in more than one group. For the features classified to be result of ISD there was one case with ambiguous mother ions (H3N4F1 and S1H4N3F1), but the intensity of ISD fragment was negligible compared to the unfragmented glycans. All the identified ISD fragments had charge state 2 and they eluted at the same time as the charge 3 mother ions with sodium and hydrogen adducts (except to one minor low intensity case, which is most likely false interpretation). Thus, there is no contradiction with the assumption that protonated (at least one hydrogen) mother ions are most vulnerable for ISD and that the resulting fragments have a lower charge state [Dr. Manfred Wuhrer, Leiden University Medical Center, private communication].

About 9 % of the total intensity was not identified. It is possible that the data still contains some glycan adducts, which were not taken into account. The potential error

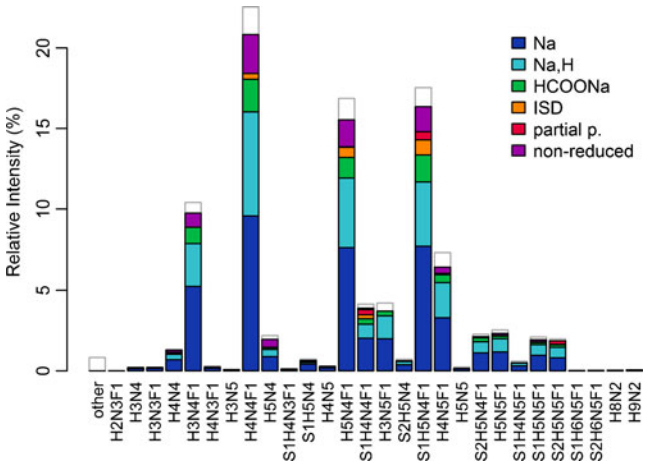

Fig. 2 IVIG N-glycan profile. Color labels are; Na: fully permethylated and reduced glycans with only sodium adduct; Na, H: as previous but with adducts with at least one hydrogen; HCOONa: glycans with sodium formate; ISD: glycans fragmented by in-source decay; partial p.: partial permethylation; non-reduced: glycans with intact reducing end. The white bar tops show the intensity of unidentified features, which have eluted at the same time as the identified glycan. Bars are scaled with total measured intensity and have been ordered according to the retention time (except the first bar 'other')

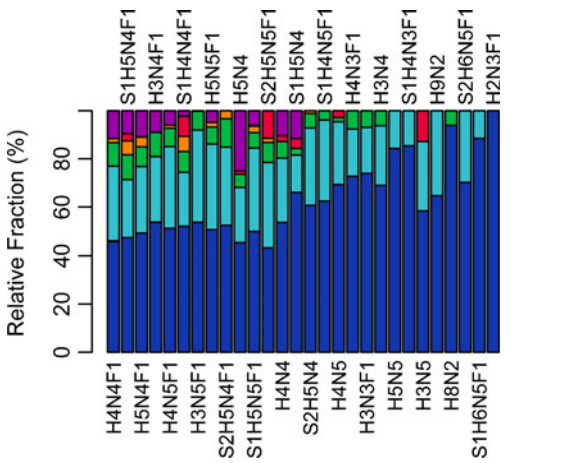

Fig. 3 Relative fractions of different adduct types for each glycan. Glycans are ordered from high (left) to low intensity (right). Colors are as in Fig. 2

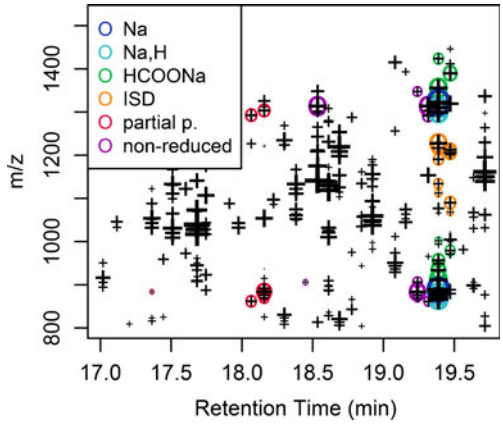

Fig. 4 Features matching to the composition S1H5N4F1. Higher m/z value and all ISD features have charge 2 and lower m/z value features charge 3. Colors are as in Fig. 2

caused by unknown variants can be estimated, if it is assumed that they do not change retention time. The amount of unidentified intensity with the same retention time (dRT <0.1 min) with the identified glycans is shown at the Fig. 2 (white bar tops) and was less than 15 % of the identified intensity to all expect four minor glycans. The exact values depend on the chosen retention time tolerance and how the intensity is distributed in case of several candidates. However, it can be assumed, that if some unassigned variants exist, they do not significantly change the profile.

The sensitivity of the automated identification for false matches was tested by adding mass shifts with integer values from −11 to 11 Da to the masses of input features and MS^2 precursors. The same acceptance criteria for automated identification was used as with non-modified data, which resulted to 23 identified compositions with 91 % total intensity coverage. For the shifted data, the highest number of matching compositions were 18 and 14 with mass shifts +2 and +11 accounting to the mass equalities of HN2=F4+2.004 and H2=NF+11.016. For the other shifts the number of matches was from 8 to 0 with an average of 3.6 compositions with 7.1 % average explained total intensity. For the non-integer shifts tested there were no matches unless the shift was close to integer value. The reason is that with the original data there were matches to almost all features with pattern, and the fact that the monosaccharide mass values have very similar decimal parts. The crude test above is not expected to give a true estimate for false positive rate. However, the false positive rate can be assumed not to be large, at least if it can be expected that the correct compositions are given in the target glycan search space.

To validate the automated feature finding performed with Progenesis LC-MS software, values for 34 features were evaluated manually. The selected ones contained all the features matching to identified glycan composition with sodium adducts (charge 2 and 3) and with matching pattern. The manual validation contained inspection of the feature monoisotopic mass, charge, retention time and total intensity (within 2.2 Da window) calculated as an integral over the eluted chromatographic peak. The analysis was done using Xcalibur (version 2.07, Thermo Scientific Ltd) and R software. The resulting monoisotopic masses and retention times were close (average mass and time differences were 0.4 ppm and 0.08 min, standard deviations 0.38 ppm and 0.06 min) and charge states were the same as given by Progenesis LC-MS. The normalized total intensity was comparable (average intensity difference 2.3 % and standard deviation 4.2 %) to all except six small intensity features. Those anomalous features (absolute difference from 11 % to 26 %) did not have well defined chromatographic peaks or they overlapped with other features, which was not taken into account by the relative crude manual analysis. Composition identifications for those 34 features were also verified by comparing to matching compositions given by GlycoWorkbench.

The extent of in-source dissociation was tested with a purified glycan sample of four oligosaccharides (H7N2, S1H5N4, SHNF and H7N6) used for the instrument calibration. The analyzed data contained four features identified to be ISD of the glycan S1H5N4. The features matched to partially permethylated compositions S1H4N4 (with 2Na, NaH and 2H adducts), H4N4 (2Na) and S1H4N3 (2Na). They had all charge 2 and were eluted within 0.1 min RT window relative to S1H5N4. The amount of ISD fragments was 5 % relative to total intensity of S1H5N4 and 8.3 % relative to unmodified S1H5N4 with sodium adducts. The glycan S1H5N4 had charge 2 and charge 3 features and the mass accuracy of ISD fragments was from −2.26 to −3.18 ppm, comparable to the accuracy of the other matching ions. For the other oligosaccharides except S1H5N4 the ISD was absent or unclear.

Fibroblast cell surface N-glycosylation

The cell surface proteins of intact human dermal fibroblasts were labeled with biotin and further captured with magnetic beads after cell lysis as previously described [18]. The N-glycans of enriched cell surface proteins were further released enzymatically, reduced with sodium borohydride, and permethylated.

The permethylated N-glycan data analyzed using LC-MS-MS^2 revealed 918 features and 1,724 MS^2 precursors within the chosen RT range (14–24 min). By mass matching alone there were 332 different compositions based on feature data and 205 on MS^2 precursor data. By analyzing the glycan groups, a total of 51 compositions were identified, 24 suggested by the workflow, and additional 27 by picking the

highest scoring ones from the remaining groups. The profile of the identified glycan compositions is shown at the Fig. 5.

For all the measured features the coverage of the identified glycans (including contaminants) was 54 % by the number and 88 % by the total intensity. Total 39 features were found to have pattern and all except one was identified. The number of features with pattern was lower than with IgG, most likely caused by the smaller intensities.

The abundance of identified non-reduced glycans was low compared to IgG sample, most likely resulting from a more complete sample reduction. The identified ISD had highest abundance among few glycans having H5N4 backbone, while the abundance of the ISD of the larger glycans was very small.

Some fully permethylated and reduced glycans eluted with two retention times suggesting possible structural differences. The most evident ones were S1H6N5F1, S1H6N5, H6N5F1 and H6N5, which had about 1 min retention time difference between two eluting groups. The exact nature of differences remained unclear. In cases where there were MS^2 spectra available from both groups the differences in MS^2 spectra were small and too incoherent to be able to deduce the structural differences.

Discussion

The rapidly growing field of glycomics has shown that glycans play key roles in biological processes ranging from development to immune defense, as well as in disease progression, and pointed attention to the potential use of glycosylation in therapeutics and diagnostics. However, the lack of robust analytical methods, and especially easy to use tools for data analysis, are still hindering progress in the field. We demonstrated here the use of in-house developed glycan analysis software *GlycanID* in combination with existing proteomic software (*Progenesis LC-MS, Nonlinear Dynamics; Mascot Distiller, Matrix Science*) for the analysis of glycan LC-MS-MS^2 data by using the N-glycans of IgG and human fibroblast cell surface proteins as examples.

In the *GlycanID* workflow the identification of high abundance glycans appear to be rather straightforward. They all have a similar simple pattern consisting of series of ions with Na and H adducts, with the ions containing only Na adducts being the most intensive. This pattern allows extracting potential (high abundance) glycan signals out of the feature data in advance before glycan mass matching. If all the patterns can be mapped to glycans it increases the confidence that there are no major unidentified glycans hiding in the data. Other members, like sodium formate adducts, could also be included in the pattern definition, but it would also limit the number of features matching the pattern, as the lower intensity ones would not be included. However the presence of those adducts increases the confidence of the identification especially if the amount is comparable to other glycans with similar intensity (and *vice versa*). For low abundance glycans there is typically no pattern available, as those glycans cannot be detected as a hydrogen adduct. For those glycan compositions the identification is based on the total mass only, and it is expected to be less reliable, especially if there is no good quality MS^2 data available.

The search strategy, to search first only glycans with sodium adducts followed by other adducts against the already found ones, proved to be beneficial. If all the variation would have been searched independently, it would have increased the number of false matches considerably, making the analysis much more complicated. Naturally, this is justified only if it can be expected that the original assumption, that all glycans appear at least once with sodium adduct, is correct, which is true at least in the setup used in the present study, where minor molarity of NaOH is added into the LC solvent phase. In the case of doubt a good strategy would be to do repeated searches with a new set of assumptions against data where previous reliable matches have been removed.

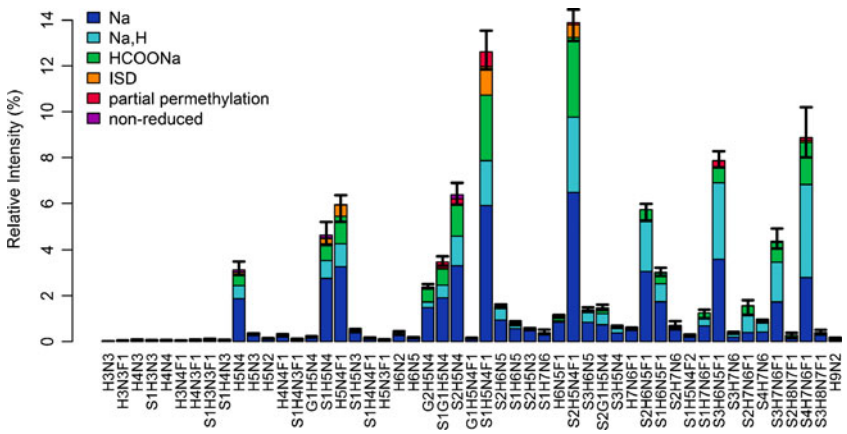

Fig. 5 Human fibroblast cell surface N-glycan profile. Error-bars show the min and max values of six repetitions. Bars are scaled with total matching intensity and have been ordered according to the retention time. Colors are as in Fig. 2

If the data complexity can be used to increase the reliability of glycan identification, that is not the case with the glycan profiling, where the total abundance of a specific glycan composition needs to be shown. The intensity has to be collected from several sources, meaning in practice sum of different features, and this poses a potential accuracy problem. The problem is most likely worse for the low intensity glycans, as all adducts may not be visible due to the detection threshold and possible false interpretations may have large effect on the total intensity. False interpretations may arise especially from partially permethylated and non-reduced glycan features as they are not tied to given RT values, but elute differently in the LC separation due to their differential chemical nature. Partial permethylation is known to occur and its level is directly related to glycan structures being permethylated [19]. With more branched and larger glycan structures permethylation is less efficient, however, the percentual amount of partial permethylation in this study never exceeded 20 % and was even considerably lower with most glycan structures. As the level of partial permethylation is dependent on the glycan structure, it is preferred to combine the intensity of partially permethylated features and fully permethylated ones. Another confusing agent in the glycan analysis is the plausible in-source decay (ISD), where the glycan is fragmented already in the source of the mass spectrometer. This phenomenon may lead to ambiguous results in case of several possible source candidates often mixed with partially permethylated glycan features, although the glycan feature resulting from the ISD has typically the same RT as the precursor from which it is derived whereas in the case of partially permethylated glycan the RT most often varies. Permethylated glycans are thought to be stable as sodium adducts in MS analysis and also in this study the measured amount of ISD was relatively small. Particularly, special caution has to be paid when interpreting MS^2 spectra with proton adduct ions because monosaccharide rearrangements or internal residue loss have been reported to occur with proton adduct ions [25, 26]. But overall we would like to claim, that in the present setup and study the possible misinterpretations, due to either partial permethylation or ISD, are expected to have only minor effect on the glycan profile.

In the present study *GlycanID* was used in the glycomic analysis of intravenous immunoglobulin, a widely used therapeutic protein drug, and of more complex N-glycan mixture from the surface of human fibroblasts. A comprehensive table of the glycans that occur on IgG and Fc and Fab fragments can be found at http://www.proglycan.com/IgG_Table_Rosetta.pdf. When the results of N-glycan analysis of purified immunoglobulin were compared to the published N-glycan profile (Fig. 6) of the plasma derived IgG, the same glycans appeared as the high abundance ones, but there were also some clear differences in the relative

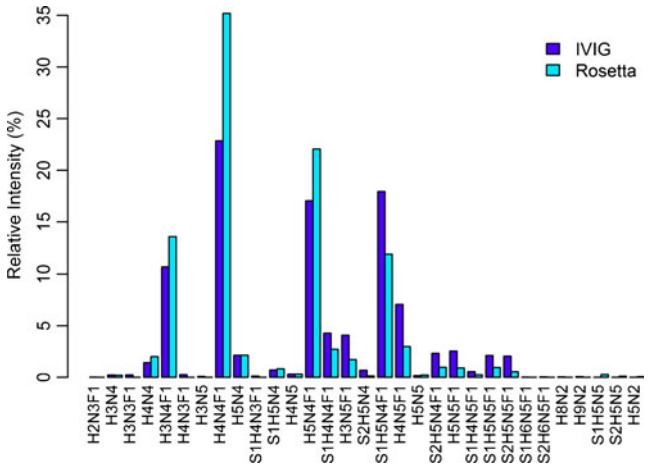

Fig. 6 Measured intravenous IgG (IVIG) N-glycan profile compared to the known profile [http://www.proglycan.com/IgG_Table_Rosetta.pdf]. Bars are scaled with total matching intensity and have been ordered according to the retention time (except the last three glycans not measured in this study)

abundances. The differences were large enough that they cannot be caused by the possible data handling errors, but have to be a consequence of different measurement techniques or inherent to the sample. The most, if not all, differences result from slightly higher abundance of sialylated and larger N-glycan compositions in the analysis of this study. This might reflect the utility of the present method in the measurement of sialylated glycans, which are inherently difficult to detect due to their labile nature. In a recent interlaboratory study, in which comparative quantitation of N-glycans of IgG and some other glycoproteins was studied, there was a surprisingly large variation in values for the degree of sialylation. The techniques employed by the participants were MALDI-TOF and HPLC of fluorescently labeled glycans. Some disadvantages are known in the determination of sialylation by these techniques, for example some sialic acid loss is reported to occur by reductive amination [27] and in MALDI process underivatized sialylated oligosaccharides are prompt to in-source and post-source decay [28]. In the present study a different analytical strategy was chosen and the N-glycan profile of IgG was measured by RP-based LC-MS-MS^2 of permethylated N-glycans, resulting a sialylation degree, which was near the upper level reported in the interlaboratory study.

Glycosylation of the IgG molecule has a profound impact on its biological functions. Artificially deglycosylated IgG is devoid of effector functions [29]. The nature of the N-glycan in the Fc portion of IgG modifies its capability to exert various effector functions. Lack of core fucosylation and presence of bisecting GlcNAc enhance the binding of Fc receptors and therefore make the antibody more cytotoxic [30, 31]. Increasing the degree of galactosylation enhances the binding of C1q and thus

complement activation [32]. The immunomodulatory effect of IgG, which is the therapeutic mechanism of IVIG, is mediated through sialylated N-glycans, which represent a minority population of N-glycans in the Fc portion [2]. The immunomodulatory effect can be enhanced by enriching sialylated forms or enzymatically sialylating IgG *in vitro* [33]. The awareness of the importance of glycosylation has raised interest in the engineering of therapeutic IgG:s to achieve maximum therapeutic effect [34], as well as called for the development of methods to analyze antibody glycosylation [35, 36].

Cell surface glycan profiling has lately proven to be useful tool in cell biology, especially when analyzing cells aimed at therapy and thus carrying out their function mainly through cellular interplay, quite often mediated by cell surface glycan structures. We have recently analyzed the N-glycome of mesenchymal stem cells [4] as well as identified more specific glycan epitopes in these cells [37, 38]. Here we demonstrate the usefulness of the *GlycanID* tool in the glycome analysis of primary fibroblasts, one of the end products of mesenchymal differentiation. Little reference data is available for fibroblast cell surface glycans, although some glycan analytics have been performed on mouse fibroblasts (Consortium for Functional Glycomics, Request 1,185) and membrane fraction from human fetal lung IMR-90 fibroblast cells [39]. Our analyses revealed the fibroblast cell surface N-glycan profile to be quite complex, consisting of several types of biantennary, triantennary and tetra-antennary species, most of which were sialylated and core fucosylated. More than 50 glycan compositions were identified, most of them being complex-type N-glycans, while some minor intensity high-mannose and hybrid type N-glycans were also detected. Comparison to the previously published fibroblast glycosylation data reveals some striking differences. High-mannose structures were abundant in IMR-90 fibroblasts, for example H9N2 was one of the most intense structures, while in the present study it was one of the minor intensity glycans. Further, in the same work with IMR-90 fibroblasts, the only sialylated species found were monosialylated ones, while here in the cell surface glycome of normal human dermal fibroblasts there were many abundant multisialylated N-glycan species, even tetrasialylated ones. It is presently unclear whether the differences are due to different fibroblast cell preparations, as the origin of the fibroblasts is different (human fetus *vs* adult dermal fibroblasts), or whether they are related to methodological differences, such as the cell surface enrichment protocol and especially different analytical workflows. However, the present method seems to have excellent performance with multiply sialylated large N-glycans, the analysis of which is known to be challenging with many other methods. We have carried out a comparison of glycan profiles measured with MALDI-TOF to the data from the present RP-based LC-MS method for some cell types, and the results have been similar, sialylated large N-glycans being slightly more abundant with the latter method (data not shown).

In a recent multi-institutional study designed to compare and evaluate various glycan analysis methods differing in sample preparation and analytical modes [28], one of the main conclusions was that MS of permethylated oligosaccharides yielded quantitative results. Permethylated glycans have been studied extensively with MALDI, but surprisingly few LC-MS methods have been developed for these compounds. In many proteomics laboratories, reversed-phase LC-MS is commonly carried out for the analysis of protein digests, but only few methods have been reported for glycans [40, 41] and improvement of these are desired. Graphitized carbon is a popular stationary phase in the LC of underivatized glycans due to its excellent isomeric separation capability [42] and its use with permethylated glycans has also been reported [16]. However, the suitability of graphitic carbon for the LC of permethylated multiply sialylated species is uncertain due to high retention and broader peaks, and this may limit the use of the method. In the present study the use of nano-scale reversed-phase separation of permethylated glycans enabled simultaneous quantification of both neutral and acidic species in the positive-ion mode, and even large multisialylated compositions could be measured with a high sensitivity.

We have been able to show here the resolving power of the in-house developed glycomic tool *GlycanID* with complex glycomic data generated using LC-MS-MS^2 technology on permethylated glycans, both from a purified glycoprotein as well as of more complex N-glycan mixture from the human cell surface. Therefore, we present the possibility to use the developed tool in the widely expanding field of glycomics, *e.g.* for therapeutic proteins as well as in studying cellular interactions.

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