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Relative quantitation of multi-antennary *N*-glycan classes: combining PGC-LC-ESI-MS with exoglycosidase digestion

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In the search for *N*-glycan disease biomarkers current glycoanalytical methods may not be revealing a complete picture of precious samples, and we may be missing valuable structural information that fall outside analysis windows. We report a targeted strategy combining PGC-LC-ESI-MS with exoglycosidases to improve the relative quantitation of tri and tetra-antennary glycan classes.

Protein glycosylation is a common and important post-translational modification that plays a key role in a wide range of biological processes. Glycans are not only essential to protein folding, immune regulation, virus-receptor binding, and developmental disorders but are a universal feature of malignant transformation and tumour progression.[1-6] In eukaryotic cells such aberrant changes are controlled by a series of glycosidases and glycotransferases along the ER–Golgi–plasma membrane trafficking pathway, highlighting the importance of comprehensive glycan profiling and relative quantitation.

To fully understand the functional roles of glycans and glycoproteins it is vital to gain an insight into the complete repertoire of oligosaccharides present. The accurate comparison of glycoforms and relative quantitation of oligosaccharides are necessary steps in this direction, importantly determining not only the monosaccharide composition of each glycan in a complex mixture but the number and abundance of each isomer is required for in-depth structural characterisation of protein glycosylation. Structural analysis of complex N-glycans is challenging due to the high level of microheterogeneity of glycan isomers and corresponding difficultly of separation.[7-9] With the advancement in technologies, there are several analytical platforms to characterize glycans and measure alterations in their abundance during pathophysiological development, which can lead to the identification of biomarkers and therapeutic targets as well as being used in the quality control of recombinant glycoproteins. Many of these technological advances have been realized by using mass spectrometry (MS), particularly matrix-assisted laser desorption ionisation and electrospray ionisation, which have emerged as powerful analytical techniques especially when coupled to online separation methods such as U/HPLC. The availability of analytical methods amenable to high-throughput and large-scale investigations is beneficial, however, dependent on sample preparation and chromatography conditions the technique results can vary and specific structural features may be under-represented or assumed from biosynthetic rules. [10-12]

Porous graphitized carbon (PGC) chromatography in combination with ESI-MS/MS detection is a versatile and sensitive tool for the analysis of native and reduced glycans and, with some limitations, also can resolve glycoconjugates including small acidic glycopeptides. PGC affords several benefits compared to amine/amide and anion-exchange phases, such as the ability to separate neutral and acidic glycans within a single run, and stability over a large pH range.

The resolving capability of PGC requires that released oligosaccharides be reduced to overcome the increased

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complexity caused by the capacity of the carbon matrix to separate anomeric forms of native sugars.[13] Furthermore, detailed structural information can be derived from elution patterns that are specific to isomeric species, in which glycans exhibiting the same mass can be separated, analysed by MS and MS/MS and their structures deduced from diagnostic fragment ions, often in combination with exoglycosidase treatments.[14] Importantly, PGC chromatography is capable of resolving structural isomers; for example, the 6-arm and 3-arm isomers of monogalactosylated biantennary glycans can be separated by PGC, while their distinction can not be easily achieved by standalone MS, often requiring tandem MS analysis interpretation.[15, 16] In addition, other structural characteristics are known to influence elution behaviour e.g., sialyated alpha(2-3)-linked structures elute later compared to alpha(2-6)-linked structures.[17] This separation feature is not only limited to sialylation, but has been reported for the differentiation and characterisation of fucosylated *N*- and *O*-glycans, in particular, Lewis-type motifs.[18] The combination of chromatographic profiling with retention time rules and tandem MS libraries are now allowing rapid and accurate identification of *N*-glycan structures that substantially aid glycomarker discovery projects.

Owing to the complexity of oligosaccharides, detailed structure characterisation often requires an orthogonal approach, such as a combination of specific exoglycosidases, to delineate and validate linkages.[19] The availability of curated databases, for example UniCarbKB and the spectral database UniCarb-DB supported by the exoglycosidase tool GlycoDigest are facilitating data interpretation.[20-22]

Relative quantitation is an essential aspect of a comprehensive glycomics study, which allows for the sensitive monitoring of glycan changes correlated to different biological conditions and diseases.[23-25] The excellent separating power of PGC permits such quantitation, and by expressing relative abundances as a percentage of the total glycans in the sample it is possible to monitor changes during disease progression. Consequently, this strategy has been used to deliver specific structural information for clinical research, such as cancer biomarker discovery, as well as in-depth comparative analysis of recombinant glycoproteins. Although current methods allow for optimal detection of high mannose and bi-antennary structures, highly branched and extended *N*-glycans may be under-represented in complex samples. This is exemplified by recent studies, which report predominately high-mannose, hybrid and complex bi-antennary structures with lower levels of more complex structures.[26-28] The relatively low reported abundance of elongated tri- and tetra-antennary structures, combined with the increasing popularity of this technology for relative quantitative glycomics comparisons, makes it necessary to evaluate its accuracy for absolute quantitation of the component glycan structures for subsequent biological interpretation.

In this study, we have (a) assessed the accuracy and precision of the current PGC-LC-MS/MS method for the detection and relative quantitation of complex *N*-glycans; and (b) identified the potential pitfalls that compromise the applicability and attainable dynamic range of PGC separation of *N*-glycans. By using released glycans from α-1-acid glycoprotein and commercial standards we have determined optimal experimental conditions for the more accurate quantitation of tetra-antennary glycans in a complex *N*-glycan mixture relative to high mannose and other complex *N*-glycan structures. Our data suggest crosstalk between interfering factors resulting in quantitative underestimations of relative amounts, and that these interferences are dependent on sample complexity. In light of our results, we propose strategies for PGC data analysis that could routinely improve interpretation of glycomic data sets, and move closer to absolute quantitation whilst also complementing existing workflows. We address PGC separation, elution and mass detection windows in the quantitation of tetra-antennary glycans in a complex mixture.

Mixtures of reduced *N*-glycans were analysed using routine PGC-LC-MS/MS methods and the relative abundances of each glycan calculated.[13] To minimise possible bias due to injection order and/or instrumental drift, samples were injected using the same solvents, over the course of a single instrument session. We found that our current method parameters have a strong bias towards high mannose and bi-antennary structures and severely under represent tetra-antennary structures. In order to demonstrate the under representation of neutral tetra-antennary glycans analysed by our standard PGC-LC-ESI-MS analysis parameters we first injected an equal molar mixture (10 pmol of each analyte) of three standard N-glycans; Man 8 (M8), galactosylated bi-antennary (A2G2) and fully galactosylated tetra-antennary (A4G4) structures. The relative abundance of each glycan structure was then calculated by integration of the area under the curve of smoothed extracted ion chromatograms for each individual mass.

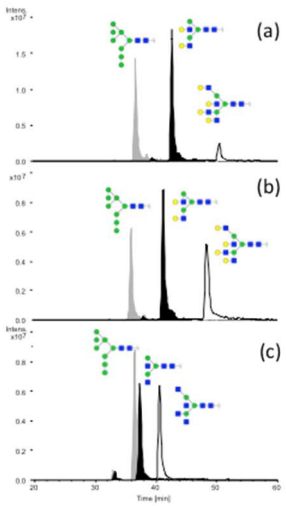

Figure 1 Comparison of the relative abundance of a mixture of three reduced *N*-glycans standards M8 (*m/z* 860.3), A2G2 (*m/z* 820.3) and A4G4 (*m/z* 1185.9) (10 pmol of each glycan). An overlay of PGC-LC-MS extracted ion chromatograms of the glycans shows a difference in relative peak abundance as detected using two different target mass settings (smart parameter setting) (a) *m/z* 900 and (b) *m/z* 1350. (c) After treatment with β(1-3,4)-galactosidase (bovine testis; BTG) the digestion products M8 (*m/z* 860.3), A2 (*m/z* 628.3) and A4 (*m/z* 861.3) detected using *m/z* 900 target mass window show expected and comparably equal

relative abundances of the products. Shorthand nomenclature (M = mannose, A= GlcNAc, G= galactose, based on the Oxford format.[29, 30]

Figure 1a shows an overlay of the extracted ion chromatograms generated for each glycan mass. Even though the glycan standards have been injected at equal concentrations, and there should be no bias in ionisation efficiency as all three structures are neutral, the intensity of the tetra-antennary structure (m/z 1185.4) is considerably lower compared to the bi-antennary (m/z 820.3) and high mannose (m/z 860.3) species. Comparison of the calculated relative percentage abundance represented as an average of replicate injections for each glycan standard is shown in Figure 2 panel A. The average abundance of M8 is 31% compared to 61% and 8% for A2G2 and A4G4 respectively. This inconsistency in relative abundance could be due to a number of factors including the mass detection window and percentage of acetonitrile at elution time.

For our approach using the Agilent Smart Parameter Setting (SPS), the acquisition mass window is set to the mid-range masses of a complex mixture; that is, to detect doubly charged *N*-glycans in the mass range *m/z* 600-1200. Consequently, glycan species close to or outside of these mass limits are poorly detected. To determine whether the acquisition mass window triggers the bias towards bi-antennary structures we shifted the target mass from m/z 900 to m/z 1350 and analysed the same standard glycan mixture, Figure 1b. By shifting the target mass window to m/z 1350 the relative percentage intensities of A4G4 (tetra-antennary) increased from 8% to 35%, the A2G2 (bi-antennary) decreased from 61% to 43%, and M8 decreased from 31% to 25%. Given the exceptional differences in relative abundances at the different target mass windows it is clear that the method and instrument setup significantly affect the relative quantitation and overestimate the bi-antennary structure.. This data may explain why many *N*-glycan studies, using PGC-LC-ESI-MS/MS, report relatively high percentages of high mannose and bi-antennary *N*-glycans.

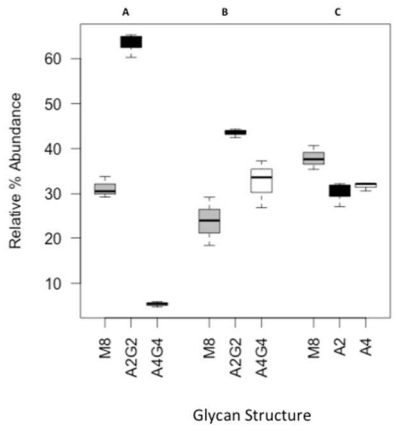

Figure 2 Box plot representing the relative percentage abundance of glycan standards M8, A2G2 and A4G4 measured at; (a) standard target mass detection settings *m/z* 900, (b) high mass detection window *m/z* 1350 and (c) digest products M8, A2 and A4 after treatment with BTG measured with standard settings. Relative abundance of each structure was calculated by integration of smoothed extracted ion chromatograms.

Relative quantitation of specific scaffold structural features can be performed with an array of exoglycosidases, that is, the sequential application of specific exoglycosidases to cleave terminal monosaccharides from the non-reducing end. By trimming complex glycans back to the antennae GlcNAc residues the total relative abundance of tetra-antennary glycans can be compared to mono-, bi-, tri-antennary species as well as hybrid and high mannose glycans.

We determined whether the existing analytical method is better suited for the relative quantitation of structures without the terminal galactoses. The standard equimolar glycan mixture was treated with beta-galactosidase (to remove terminal galactose residues) and the relative abundance calculated for each structure. This approach will collapse glycan structures with the same branching core into single neutral peaks, simplifying detection and quantitation, Figure 1c shows an example as an overlay of extracted ion chromatograms for the beta-galactosidase digest product masses of the two galactosylated standards; the parent and intermediate product masses were also monitored for any evidence of incomplete digestion. After digestion the relative percentage abundance of the bi- and tetra-antennary structures become equivalent with a slight bias toward the high mannose glycan. By cleaving terminal galactose residues and exposing the antennae GlcNAc residues we have collapsed the masses not only into the optimal mass detection region but also into a smaller elution window, which means any influence of the percentage of the acetonitrile organic solvent are minimised.

Figure 2 summaries the relative abundances calculated for each glycan standard obtained from the three methods i) standard target mass detection at m/z 900 ii) shifted detection window (m/z 1350) and iii) exoglycosidase treatment. It is clear that the true abundance of each standard glycan is best measured after exoglycosidase digestion using the target mass setting (m/z 900) and gradient i.e. the data shows a smaller level of variation for the percentage abundance of each structure in Figure 2 panel C.

To further validate the quantitation of complex glycan standards by PGC-LC-ESI-MS/MS we analysed a suitable glycoprotein standard, α1-acid glycoprotein. α1-acid glycoprotein is well characterised and reported to contain a mixture of fully sialyated, bi-, tri- and tetra-antennary glycans. Reduced *N*-glycans released from α1-acid glycoprotein were analysed and the bi-, tri- and tetra-antennary glycan classes (without outer arm fucosylation) were quantitated before and after exoglycosidase treatment. Extracted ion chromatograms were generated for the sialyated oligosaccharides and the digested products following sialidase and galactosidase treatment (Figure 3a), and the combined relative abundances compared. After sialidase treatment the data shows that bi-antennary glycans are the abundant species (~70 %) followed by tri-antennary (~20 %) and tetra-antennary (~10 %), Figure 4b. These results differ from previous reports detailing the analysis of α1-acid glycoprotein 2AA-labled *N*-glycans by MALDI-TOF where tri-antennary structures were of highest abundance.[31]

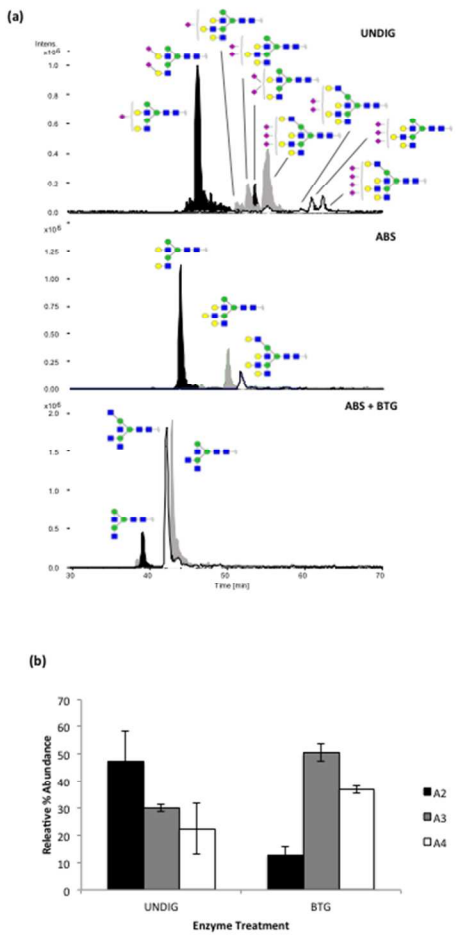

Figure 3 N-glycan structures released from alpha-1 acid glycoprotein analysed by PGC-LC-MS before and after treatment with exoglycosidase enzymes. (a) Overlay of extracted ion chromatograms for glycan masses corresponding to bi- (black), tri- (grey) and tetra-antennary (white) structures. (b) Relative percentage abundance of combined bi- (A2, black), tri- (A3, grey) and tetra-antennary (A4, white) glycan structures before and after enzyme (sialidase + galactosidase) treatment, mean and standard deviation of triplicate release are shown.

Quantitation of bi-, tri- and tetra- antennary glycan structures after cleavage of terminal sialic acid and galactose residues again shows a very different relative distribution. Analysis of released glycans, trimmed back to the GlcNAc antennae, shows a higher proportion of tri- (~50 %) and tetra-antennary (~35 %) structures compared to bi-antennary (~15 %), Figure 3b. By collapsing the structures to the truncated antennary form the glycan masses are shifted closer into the optimal target mass setting (*m/z* 900), thereby allowing for a more accurate determination of relative abundance. Notably, unlike HILIC-U/HPLC combined with florescence detection, which gives consistent abundance measurements of glycan classes before and after exoglycosidase digestion based on the fluorophore attached to the reducing end, we have shown that the abundance of glycan classes detected by PCG-LC-MS can vary significantly dependent on the exoglycosidase panel.[32, 33] Therefore care must be taken when choosing conditions for reporting quantities of glycan features within a complex biological sample.

Finally, the *N*-glycan profiles of membrane proteins from the melanoma cell line MM253 were analyzed (Figure 4). Here, we compared the relative abundance of *N*-glycans before and after exoglycosidase(s) treatment. It is clear (Figure 4b) that the tri- and tetra-antennary *N*-glycans are under-represented in the undigested sample set and that the data shows a bias to the high mannose family. A three-fold increase in tri- and tetra-antennary structure classes is gained after the released glycans are treated with the panel of three exoglycosidases (sialidase, glactosidase and fucosidase). The presence of outer-arm fucosylation can impede the activity of galactosidase due to steric hindrance; therefore if fucose residues are present galactosidase should be used in combination with a general fucosidase. As such, and similar to the data shown for α1-acid glycoprotein, the assignment of absolute cell surface glycosylation would be severely under estimated by solely using PGC-LC-ESI-MS/MS data.

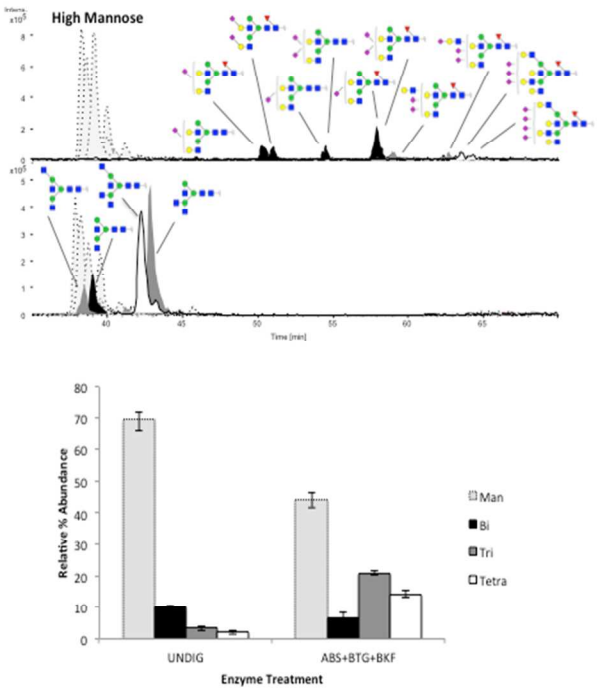

Figure 4 Membrane protein *N*-glycans released from the melanoma cell line MM253. (a) Top panel shows the extracted ion chromatograms (EIC) for high mannose, bi-, tri- and tetra-antennary glycan structures and (b) EICs for the trimmed structures exposing the antennae core after treatment with α(2-3,6,8,9)-sialidase (*Arthrobacter ureafaciens*; ABS), β(1-3,4)-galactosidase (Bovine testis; BTG) and α(1-2,3,4,6)-Fucosidase (BKF). (b) A comparison of the relative abundance of glycan classes, bi- (black), tri- (grey) and tetra-antennary (white), before and after enzyme treatment, mean and range of triplicate releases shown.

Although we have demonstrated that the use of exoglycosidase enzymes for the quantitation of highly branched structures gives a more accurate representation of the quantitative abundance of the glycan classes it is still not optimal as information on individual isomers present in the undigested sample is lost. It is well reported that glycans bearing beta 1-6 branching, α2,6 sialylation and lactosamine extensions have been implicated in cancer, many of these

studies used lectin binding analysis and/or mass spectrometry for identification and quantitation.[34] This suggests that we are missing vital information on these larger glycan classes by under representing their true abundance.

Conclusions

Analytical methods and platforms for global profiling of glycosylation and the emergence of glycoproteomics are enabling us to identify differentially expressed glycan structures during disease development. The present study highlights that no one method is suitable for every sample set and the importance of understanding the limitations of routine methods and the need to optimise analysis conditions to best represent the sample is essential. It is difficult to set optimal analysis parameters for the detection of all oligosaccharides, but with analysis moving toward integration of multiple -omic data sets, for example correlation of the glycan structural products with expression levels of glycosyltransferase genes, it is important to identify optimal parameters for data acquisition including: i) the accurate relative quantitation of each glycan structure between individuals and ii) the accurate relative quantitation of structural classes within a single sample (for instance the ratio of bi-antennary to tetra-antennary glycans). This method builds upon a widely used analytical method for glycan analysis but adds the extra dimension of exoglycosidase digestions to gain a better insight into the true abundance of glycan classes. The described platform can be readily employed for the profiling of glycan structural classes on proteins in complex biological samples and biologics.

All experiments were performed using an Agilent 1260 capillary HPLC (Agilent Technologies Inc., CA, USA) and glycan masses detected using an Agilent 6330 ESI ion trap (Agilent Technologies Inc., CA, USA). *N*-glycans were separated using a porous graphitized carbon (Hypercarb KAPPA Capillary Column; Thermo Fisher Scientific) 0.18 mm inner diameter × 100 mm 3 µm particle size, and data processed using Bruker DataAnalysis software version 4.0 (Bruker Daltonics, Germany). It is important to note that the reported impact of target mass settings on relative abundances is based on data acquired with an ion trap mass spectrometer, and that the same observations may not necessarily be observed with different detector technologies. A summary of the experimental conditions and enzyme concentrations used are provided as supplementary information and based on previously described methods[13, 19, 35, 36]. We also provide the assigned MS^2 spectra along with summary tables that show detected and calculated masses as supplementary data.

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