Rapid and sensitive methods for the analysis and identification of O-glycans from glycoproteins

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English summary

Dutch summary

Abbreviations

Curriculum Vitae

List of publications

Acknowledgements

English summary

Glycans are very diverse structures and provide a valuable source of biomarkers. The O-glycans on glycoproteins and mucins play very important roles in immunity, leukocyte trafficking, vascular biology, angiogenesis and lymphangiogenesis. Mucin O-glycans also protect gut epithelial cells from the microflora and extreme pH and prevent tumorigenesis.

Changes in both N- and O-glycosylation have been associated with many states of health and disease, providing prognostic and diagnostic information. O-glycans have been relatively little studied through lack of suitable analytical tools but the evidence is that they could be promising biomarkers.

O-glycan profiles successfully distinguished breast cancer samples from normal samples. Truncated O-glycans on the hinge region of IgA are associated with IgA nephropathy. Future studies need to further define key O-glycans whose alterations reflect disease progression. Such knowledge will be great importance critical in developing novel diagnostic and therapeutic strategies.

Over the last decade the number of approved protein biopharmaceuticals has been rapidly growing with a total sales value of $140 billion representing approximately 20% of the total pharmaceutical market. Protein biopharmaceuticals include fusion proteins, cytokines, monoclonal antibodies (mAb), vaccines, hormones, growth factors, and blood factors. More than two-thirds of these recombinant biopharmaceuticals products are glycoproteins, and glycans have been associated with safety and efficacy of biopharmaceuticals. Most of the glycoprotein therapeutics bear N-glycans but O-glycans are also found in some economically important biopharmaceuticals such as EPO. The FDA technical guidance for

characterizing and monitoring glycans says: “Glycan structures should be characterized, and particular attention should be paid to their degree of mannosylation, galactosylation, fucosylation and sialylation. The distribution of main glycan structures present should be determined.”

The aim of the work described in this thesis was to develop and improve methods for O-glycosylation analysis. Over the last decade a number of techniques for O-glycan analysis have been developed, however release of O-glycans, the first essential step of analysis, remains very challenging (Chapter 2). The main reason for this is that there is no enzyme available for universal O-glycan release from proteins. The commercially available O-glycanases, or endo-α-N-acetylgalactosamidases, have a high specificity limited to the release of neutral core 1 O-glycans (Galβ1-3GalNAc). Therefore, chemical release is the most effective method for releasing the full range of O-glycans. Several chemical methods for liberation of O-glycans have been described and the most common one is reductive β-elimination. This release methods leads to O-glycans in their reduced forms (alditols). This makes it impossible to introduce reducing-end tags for fluorescent detection and the analysis is restricted to mass spectrometry, high-pH anion-exchange chromatography with pulsed amperometric detection or nuclear magnetic resonance. Several techniques for chemical release of O-glycans in non-reduced form have been developed and it has been reported that the best method for universal O-glycan release in high yields is hydrazinolysis.

Unfortunately all of the O-glycan release methods show a side reaction known as “peeling”, which is a stepwise degradation of the polysaccharide starting at the reducing end and removing one sugar residue at a time. Peeling is a well-known

problem when performing O-glycan release and often results in poor repeatability with variable amounts of the small peeled glycans. This is a major problem for comparability studies or quality control. Biopharmaceutical developers and researchers have investigated this peeling problem. In Chapters 3 and 4 the development of sample preparation techniques for O-glycan release (in their nonreduced form) by hydrazinolysis are described. The methods have been optimized to reduce the undesirable peeling reaction. Developed techniques show that buffer exchange into 0.1% TFA or 100 mM EDTA prior to hydrazinolysis or addition of EDTA directly to hydrazine significantly reduces the amount of unwanted peeling. A drawback of previous methods was a high level (up to 70%) of this side reaction. With the methods described in this thesis, peeling product went down to 17-20% making the O-glyan analysis more robust.

One of the most widely used methods for glycan analysis is reducing-end labelling of released glycans by reductive amination followed by HILIC-UHPLC with fluorescent detection. The stoichiometric attachment of one tag per glycan allows the relative quantitation of different glycan species based on fluorescence or ultraviolet (UV) absorbance intensity. Several fluorescent tags have been used for glycan derivatization such as 2-aminobenzamide (2-AB), 2-aminobenzoic acid (2-AA), 1-aminopyrene-3,6,8-trisulfonic acid (APTS), 2-aminopyridine (AP), procaine, and procainamide. In Chapter 5 we have shown that procainamide-labelled glycans gave higher fluorescent response and significantly higher ion intensity in positive ESI-MS when compared to the most widely used label 2-AB. The procainamide-labelled glycans are suitable for both (U)HPLC–FLR analysis, providing good chromatographic separation for relative quantitation, and ESI–MS analysis, providing more efficient ionization for glycan identification.

Using the strategy described in Chapters 3 (“Suppression of peeling during the release of O-glycans by hydrazinolysis”), 4 (“Improved nonreductive O-glycan release by hydrazinolysis with EDTA addition”) and 5 (“Comparison of procainamide and 2-aminobenzamide labelling for profiling and identification of glycans by LC-FLR-ESI-MS”), combining O-glycan release by hydrazinolysis with procainamide labelling followed by HILIC-UHPLC-ESI-MS/MS analysis we studied O-glycosylation of human saliva. We used human saliva because the wide spectrum of compounds (including glycoproteins) present in saliva may provide information for potentially important biomarkers and it is relatively easy to obtain. Our study showed that the composition and relative abundance of the O-glycans in human saliva significantly changed during the day. This observation should be taken into consideration by researches evaluating salivary O-glycosylation for its diagnostic biomarker potential.

The closing chapter of this thesis (Chapter 7) contains a general discussion, which places the individual chapters into context. We believe that our technology for O-glycosylation analysis can be applied by researchers for analysis of complex biological samples and for O-glycan biomarker discovery. It is also anticipated that presented, improved technology will be used by drug developers at the early stages of drug development through to post-approval batch release. This strategy can be incorporated into drug developers’ programmes to support QbD-based realisation and regulatory submissions of glycoprotein therapeutics. However, further studies are needed to better understand and evaluate biological functions or for assessing therapeutic the efficacy of mucin type O-glycosylation.