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Glycosidases as an analytical tool in glycomics assays

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Chapter 6

Discussion and perspectives

Glycomics has proven to be valuable in clinical and medical research. Associations with disease states complement and occasionally supersede the diagnostic potential of proteomics, metabolomics or genomics [1-4]. Sometimes, glycomics is also being developed as an alternative to invasive examinations, such as in the case of prostrate [5, 6] and colorectal [7, 8] cancers. However, the analysis is often complicated by the complexity of the glycan structures which show variations in both composition and linkages of the constituent monosaccharide residues [9, 10]. The major human plasma proteins are estimated to contain at least 167 *N*-glycan structures which complicates meaningful associations of disease states to glycomics data [11, 12]. The identification and quantification of these glycans are usually performed using chromatographic and spectrometric techniques [12-15]. The compilation of glycan motifs and epitope features into so-called glycan traits has enabled an improved comparison of variations in glycosylation between patients [13, 16, 17]. These academic research successes have created an interest in glycomics as a clinical prognostic assay. Accordingly, contract research organizations as well as assay developers have entered the field of glycomics [18-21]. This thesis addresses this commercial interest by developing assays for routine industrial applications with commercial potential for the development into off-the-shelf, on-site products.

Analytical techniques in glycomics

In clinical glycomics, development of analytical techniques and medical knowledge is co-dependent. Advances in separation and identification technologies have allowed for understanding and confirming the complex mechanism of glycosylation in a living entity [4, 22, 23]. The more we understand the biology behind glycosylation, the more demand in advanced analytical techniques is developing to further push the boundaries of our understanding. Early glycomics assays were based on lectin blots which utilise the structure-specific binding activities of lectins to identify certain glycan motifs [24-26]. Around the same time, a move into performing glycomics assays on liquid chromatography (LC) - fluorescence detector (FLD) and pulsed amperometric detection (PAD) platforms gained momentum. In fact, LC-based assays still remain the main workhorses of industrial and academic glycomics. They are applied, for example, for the glycosylation characterization of individual proteins and complex biological samples, such as TPNG [13, 27, 28]. Analyte identification using this technique is based on retention times which are normalised to a glucose polymer ladder or other glycan standards which enables data set comparisons [29]. For the elucidation of glycan structures, a sequential process of exoglycosidase digestion is performed before the LC analysis [29]. By correlating the shifts in peaks between the digestion regimes, the structure of a glycan can be built up [29, 30]. Although these chromatographic techniques have been exceptionally successful in glycomics, they can sometimes be limited by their molecular resolution. This holds true for hydrophilic interaction liquid chromatography (HILIC) which is one of the most prominent LC methods used in glycomics assays. The separation performance of HILIC methods depends on the analytes partitioning between the mobile phase layers created on the polar stationary phase [31]. However, *N*-glycans have a characteristic pentasaccharide core structure, with variations mainly in the antennary branching and decorations of galactosylation, sialylation and fucosylation [9, 32]. Thus, minor differences

between glycans, such as a different linkage of a monosaccharide, may result in only minute differences of physicochemical properties making their HILIC separation challenging. This may result in several glycan structures co-eluting under a single chromatographic peak. Despite this limitation, HILIC analysis has time and time again proven to be successful in drawing associations to certain disease states in patients [15, 33]. Many of the analyses rely on comparison of the relatively abundance of the chromatographic peaks rather than the individual glycans that form a peak [34].

However, the analytical questions posed in cutting-edge medical research and biopharmaceutical development, require detailed analytical answers regarding e.g. the structure of individual glycans and their quantities [35-39]. Thus, separation techniques with improved resolutions such as multimodal LC [40], capillary electrophoresis (CE) [11] or capillary gel electrophoresis (CGE) [41] have been explored. LC and CE can be coupled to mass spectrometry (MS) devices, mainly via electrospray ionisation (ESI) – MS, which provides a second analytical dimension namely *m/z* identification. Thus, even if a chosen separation technique is unable to differentiate between glycan structures, a mass spectrum obtained may allow to identify and quantify these structures. Furthermore, improvements in ion optics over the past decades have allowed for improvements in selective gas phase fragmentations that enable structural sequencing of the analytes.

Existing glycomics assays

The importance of glycomics is growing along with our understanding of the correlations of glycomics changes to disease progression. As a result, a growing interest for glycomics is created in industry. There is only a handful of analytical chemistry companies that specialise in glycomics analysis of clinical and biopharmaceutical samples. Many of these industrial applications rely of LC and MS-based methods that screen an entire glycome. However, due to the complexity of glycomes, such as TPNG, often structures that have clinical or medical importance are unresolved. The work of this thesis primarily addresses this issue by developing novel glycomics assays that are designed to specifically quantify targeted epitopes in TPNG or IgG glycome samples. These assays thus enhance quantification of certain important glycan epitopes such as antennary fucosylation which are normally tedious to quantify by the commonly used LC-FLD/MS methods in industry. Besides, the industrial glycomics services, some of these organisations are also involved in developing kits for glycomics assays. However, many of these analytical kits are made for sample preparation such as reducing end labelling [42, 43] and methylation [44] of glycans for analysis on LC/CE/CGE-FLD/MS platforms [45-48]. The work of this thesis addresses the demand for innovative kit development by introducing an assay which does not require the latter analytical platforms but rather a spectrophotometric plate-reader. These assays could hence meet demand in laboratories that are not specialised in routine glycomics analysis. Especially since now-a-days many laboratories embark on cross-disciplinary research topics, such analytical glycomics kits could serve as a quick and efficient solution for attempting to address a glycomics research question.

Simple glycomics assays

Glycomics is heavily dependent on chromatography, electrophoresis and MS. However, unlike proteomics and genomics, not much investigation into alternative non-chromatographic assays has been done [49, 50]. It is true that detailed analytical questions in proteomics and genomics require some form of separation. However, many assays have been created as an alternative to answer certain niche and targeted analytical questions without the need for chromatography. For instance, enzyme linked immuno sorbent assay (ELISA) are often used in proteomics and quantitative polymerase chain reactions (qPCR) assays are used in genomics to quantify the abundance of certain proteins or DNA epitopes in a sample, respectively. Such plate-based assays have a plethora of advantages which will be discussed along the span of this section. An established alterative to chromatographic /electrophoretic/mass spectrometric glycomics assays for complex samples, such as TPNG, is the lectin microarray assay [51, 52]. Similar to an ELISA format, an analyte of interested is immobilised and quantified based on the binding specificity of a detection probe.

However, these microarrays are not widely adopted due to the technical skills required for assay establishment as well as multiple sample processing steps [53, 54]. Moreover, performance of the assay is heavily dependent on specificity and binding affinity of the lectin probes. A few research groups have developed another sort of a plate-based assay that is designed around redox reactions of monosaccharides units to produce a colorimetric signal [55, 56]. Building from this research, this thesis introduces the development of an assay using enzyme-based redox reactions for the quantification of glycan epitopes. In general, analytes and reactants are mixed within a microtitre plate and the colorimetric signal is measured on a spectrophotometric plate-reader [55, 56]. Thus, these assays do not require the tedious process of immobilising antibodies/lectins to the microarray. The immobilisation is a common source of variability arising from variations in immobilised lectin density and stoichiometry [54].

Critical aspects for assay development targeting industrial application

One-pot reactions

Analytical chemistry assays used in industrial applications should preferably have a streamlined workflow with minimal hands-on time. There are several reasons for these requirements, but the most important one is the operator’s ability to perform an assay with minimal training. Therefore, the assays consist of a minimal number of steps; hence one/two-pot reactions are desirable. This also reduces the necessary laboratory cost on consumables and waste. Such one pot assays usually also allow for an easy transfer of the assay to a fluidic handling robotic platforms which aids in throughput. Furthermore, such assays usually allow for concise comprehensive standard operating protocols. In chemistry, compiling several reaction steps into a one-pot reaction procedure is seldom easy, due to possible interferences between the constituents required for the different reaction steps. However, many biochemical assays such as ELISA and qPCR are based on one-pot biocatalysed enzyme-based

reactions [57, 58]. Due to the specificity of enzymes to catalyse certain reaction steps, several steps can be compiled into a one-pot reaction with little to no interference of inter-mediate reaction steps. However, since enzymes are functional proteins, a deviation from their suitable reaction conditions can limit their activity [59]. Thus, when designing such one-pot reactions with multiple enzyme catalysed steps, the physio-chemical conditions of the reaction should strike a balance between the efficiencies of the constituent enzyme activities so as not to limit the overall efficiency of the assay [60]. In case of drastic differences in the required physico-chemical conditions of the different constituent reaction steps, the individual reactions of the one-pot reaction can be performed in a sequential manner. Chapter 5 demonstrates such a sequential one-pot assay. Exoglycosidases, dehydrogenases and an epimerase were the enzyme biocatalysts used in this multi-step one-pot reaction spectrophotometric glycomics assay. By performing several reaction steps sequentially with increasing reaction volumes, tolerable biochemical conditions for enzymes were achievable whilst obvious interferences between reactions steps were limited.

Instrumentation and data

Assays based on spectrophotometric plate-readers are advantageous when it comes to the overall infrastructure and costs of an assay. These plate-readers usually cost much less than LC, CE and MS platforms, and often do not require extensive regular maintenance or skilled technicians. Modern plate-readers are also compact benchtop instruments that require much less laboratory space and no gas lines or vents as compared to LC, CE or MS instruments. Thus, a dedicated analytical service laboratory can furnish many such plate-readers easily, if demand increases. Additionally, the machine time for data analysis of a sample in a spectrophotometric plate-reader is only a fraction of the time required by separation-based platforms, which increases throughput. Their ease-of-use is highly desired for industrial analytical application, e.g enabling quick training of technical staff.

The identification and quantification ability of LC and CE techniques is based on their ability to separate analytes either in time or space irrespective of the detection system used. This is usually in contrast to assays based on spectrophotometric plate-readers. A sample containing one or more analytes of interest is placed in a single well of a microtitre plate, and these analytes are exclusively selected based on their spectrophotometric profile of the absorbed and/or emitted light. Thus, the identification and quantification ability of a spectrophotometric plate-reader assay is limited to the differences in the spectrophotometric profile of the analytes in the sample, i.e. analytes with similar or overlapping spectrophotometric profiles cannot be differentiated [61, 62]. However, physical chemical approaches using quantum dots and Förster resonance energy transfer (FRET) are being investigated to improve the multiplexing of spectrophotometric assays based on time dependence and narrow bandwidths of spectrophotometric profiles [63, 64].

LC, CE and MS quantification often rely on relative area comparison of the integrated peaks in a chromatogram, electropherogram and mass spectrum, respectively. In contrast, for spectrophotometric plate-reader assays, an absolute quantification is performed based on comparison to a standard curve which is a concentration series of the analyte of interest. The

latter can be limiting when it comes to the analysis of some biological molecules, due to the availability of standards. For example, the quantification of a glycan epitope on a specific glycoprotein is limited by the fact that pure protein glycoform standards hardly exist. When only the relative biological variation of the glycan epitopes is clinically relevant, then measuring absolute glycan epitope amounts would also incorporate the biological variation of the glycoprotein expression which may not necessarily have clinical relevance. In this case, the quantified glycan epitope would need to be normalised for the clinically irrelevant variations of the glycoprotein. One approach is to take the ratio of the glycan epitope amounts to the protein amounts, wherein the glycan epitopes and protein amounts are firstly quantified against their respective molecular standard curves. Alternatively, a relative quantification approach can also be performed by taking a ratio of two glycan epitopes that may be structurally inter-dependent in the parent biomolecule. The added benefit of a normalisation approach using the ratio of epitopes quantified by similar chemical and detection mechanisms, is the elimination of the need for a standard curve to compare between samples. For example, taking the ratio of galactosylation and sialylation for a glycoprotein can eliminate variations coming from the glycoprotein amounts of different samples. This was exemplified in Chapter 5.

Other research aspects

The assays introduced in this thesis were developed with the intention of easily transforming them into analytical kits. Besides being potential products for use in established industrial and academic laboratories, a major advantage of these kit-based assays is the portability for in-field research applications. Such kits and spectrophotometric plate-readers can be easily transported and do not require laboratory infrastructure such as gas lines and vents. Furthermore, the use of light emitting diodes (LEDs) with wavelengths of interest rather than white-light sources, has allowed for a scaling down of plate readers to the size of booklets (Absorbance 96, Byonoy GmbH, Germany) and hence has further improving portability [65].

In-field research has its advantages as it saves time for answering an analytical question but more importantly it removes the need to transport temperature-sensitive biological samples to an analytical laboratory which is not always located near a site of the field research. It must be mentioned that the assay in Chapter 5 was performed almost entirely on a Hamilton robotic system, but there is no reason why it cannot be performed similarly with multichannel pipettes, which was the case during its development.

Identification options in (LC-)MS assays

Fragmentation and glycan epitope identification

The complexity of glycomics partially derives from the linkage variants of residues. However, obtaining information on the exact loci of these residues within a molecule can further increase the complexity of glycomics analysis. Considering only linkage variations, often two or more linkage variants of a residue can occur on the same molecule. Sometimes, these linkage iterations cannot be resolved with a separation method. Thus, a coupled MS dimension helps to derive the structural features by gas phase fragmentation which, in glycomics, is usually collision induced dissociation (CID) [66]. Most MS based glycomics assays are performed in positive ion mode due to the higher (compared to negative ion mode) ionisation efficiency [66]. However, CID in positive ion mode results in glycosidic bond breaks which may not always be sufficiently informative, especially in the case of co-eluting linkage variants [67]. Furthermore, complete coverage of molecular fragmentation patterns is not always obtainable. However, if we consider *N*-glycosylation in humans, the linkage variants of clinical interest are mostly of fucoses (Fuc), N-acetylglucosamines (GlcNAc) and sialic acids (Sia), which are almost entirely *N*-acetylneuraminic acid. Linkage variants of galactose (Gal) and mannose can also be found. Each of these residues has its own generally accepted manner of identification. In case of Fuc, diagnostic fragment ions representing parts of the glycan core structure are used for identifying its α(1-6) or α(1-3/4) variants which are exclusively core or antennary located, respectively [68]. Similar diagnostic ions representing part of the core can be used for differentiating between β(1-4) bisecting GlcNAc and β(1-2/4/6) antennary GlcNAcs [69]. However, differentiating between the linkage variants of antennary GlcNAcs by CID fragmentation is particularly difficult.

In the previous paragraph, we only considered the identification of linkage variants without considering the loci of these variants within a molecule. It is worth mentioning that glycans having the same composition including their constitutional isomers, may not necessarily have the same structure because the residues of antennary GlcNAc, Gal, antennary Fuc and Sia can be assorted onto different antennary arms. The possibility of the presence of such regioisomers becomes especially concerning in glycans with multiple antennae. CID in positive ion mode is unable to provide sufficient information to elucidate the position of such residues. However, CID in negative ion mode results in sequential cross-ring fragmentation patterns from which such information can be derived [67]. This makes this method a very powerful tool in glycomics analysis. However, the drawback is that ESI of glycans in negative ion mode suffers from low ionisation efficiencies. Furthermore, fragmentation data is not always easily comprehensible and hence negative ion mode analysis is not widely practiced in glycomics.

Of special relevance to this thesis are the diagnostic fragment ions that are used for differentiating the linkage variants of Fuc residues due to their core or antennary localisation on the glycan structure. However, these diagnostic ions are not conclusive due to the phenomenon of Fuc rearrangement. The α(1-3/4) antennary Fuc residues have a tendency to undergo a gas phase molecular rearrangement to form isomeric ions, mimicking core fragment ions [70]. Considering that antennary fucosylation in TPNG has been associated with several cancers [27, 71], a demand for its accurate quantification has been created. In aid of

improving the accuracy of antennary Fuc quantification, Chapter 4 takes a different approach to add selectivity. The novelty of this assay lies in its ability to accurately quantify antennary fucosylation in complex TPNG samples without the use of MS fragmentation. The assay relies on an exoglycosidase depletion of core Fuc residues so as to obtain information on the antennary Fuc residues by matrix assisted laser desorption ionisation (MALDI)-time of flight (TOF)-MS. This allows for the calculation of antennary Fuc traits without the need for complex and sometimes inconclusive MS/MS fragmentation experiments. Thus by taking advantage of the selectivity of exoglycosidase, the need for technical and experimental complexity can be reduced in glycomics. This approach has potential in differentially branched antennary GlcNAc isomer quantification where MS/MS experiments required for their identification are very complex. This is why a portion of the work of this thesis was dedicated to characterising the substrate specificities of exoglycosidase, which could have potential not only for glycan profiling but also for similar aforementioned exoglycosidase-based analytical assays. More of this will be discussed in the later section.

It is also worth mentioning, that when it comes to analytical speed, MALDI-MS methods are faster than LC-ESI-MS methods on which most antennary Fuc assays are based. This makes the assay even more attractive for an industrial application as throughput is often vital.

Derivatization

Few years ago, identification of Sia linkages by fragmentation was very challenging due to its terminal location at the non-reducing end and lack of differentiating diagnostic ions. However, derivatisation methods such as methyl esterification [72] and ethyl esterification, and it adaptions [73-75], have greatly enhanced identification of the most prominent α(2-3/6) Sia variants. During these esterification reactions, a methyl/ethyl ester is preferably formed on the C1 -COOH of α(2-6) Sia rather than α(2-3) Sia, due to their differential relative proximity to the C2 -OH of the penultimate galactose (Gal) residue [73]. This reaction selectivity causes a differential mass shift between both linkage variants. As a result the composition of the Sia variants within a structure can be identified even without the need for fragmentation [76]. Such chemical derivatisation approaches have not been investigated for Fuc and bisecting/antennary GlcNAc residues, and could possibly be very tedious due to the lack of unique and suitable chemically active functional groups in these residues. Thus, exoglycosidase-based glycan profiling still remains the industrial gold-standard for identifying Fuc and GlcNAc residue linkages. Hence, obtaining fucosidases and GlcNAc-ases of isomer specificity is important.

Exoglycosidases for analytical use

The performance of the analytical assays developed in this thesis as well as other glycoprofiling techniques are highly dependent on the specificity of the exoglycosidases used. Hence, it is important that these glycosidases are well characterised in terms of substrate specificity so as to ensure high accuracy in quantification of isomeric analytes. Glycosidases have an ever growing demand in bioindustry and academic research applications. Hence, many of these glycosidases are readily obtainable from commercial vendors as recombinant or naturally sourced products. Furthermore, molecular biologist with experience in gene expression can produce these recombinant glycosidases in-house. However, the source of the glycosidases is highly dependent on the user preferences and reliability of the enzymes available. This can be exemplified by the choice of the sources of glycosidases used in the assays developed in this thesis. These glycosidases are either commercially available or can be produced readily by molecular biologist in-house as recombinant proteins. Commercially available enzymes are usually well characterised for specificity and kinetic activity and hence ideal for assay development as it removes the need for enzyme characterisation. In an academic sense, availability of commercially sourced enzymes also allows the assay to be reproduced readily by other research groups that are interested in the topic. This was one of the underlying motives in Chapter 4, were a commercial core fucosidase was used rather than the non-commercially sourced recombinant core fucosidases which were also available. However, commercial enzymes can sometimes be quite costly. Since the assay was intended for cost effective high-throughput analysis, the use of in-house sourced recombinant exoglycosidases was ideal rather than the more expensive commercial options. Many of these exoglycosidases can also be inexpensively commercially available as natural sourced enrichments. These naturally sourced enzymes usually have one or more purification steps for enriching the enzyme of interest. Since these purification steps are often limited in purification efficiency, glycan and/or other exoglycosidase contaminants could introduce variations in the assay when using difference batches of the enzymes. Hence to avoid such variations, recombinant enzymes of high purity should be used.

Exoglycosidases of potential analytical interest

Although there is a large repertoire of available glycosidases, there is still a demand for certain glycosidase activities which can be based on specificity to a certain linkage or on a certain glycan structure. There will always be a demand for obtaining glycosidases of certain specificity which depends on the needs of a glycomics assay or a glycobiological application. Enzymology is a large research field and so to convey the importance of glycosidases in glycomics, I shall exemplify from the work done in this thesis on an antennary fucosidase (Chapter 2) and a bisecting GlcNAc specific GlcNAcase ([77], Chapter 3). These enzymes were chosen for screening due to difficulties in identifying their substrate glycan isomers by analytical glycoprofiling techniques. As mentioned above, identification of isomeric fucose residues on *N*-glycans is tedious to due to unreliable diagnostic fragment ions. Similar diagnostic fragments ions of bisecting GlcNAc isomers are usually present in low abundance along with the low occurrence of these bisecting glycans in TPNG [78, 79].

The antennary fucosidase here specifically removes α(1-3/4)-linked antennary Fuc residues irrespective of sialylation on the Lewis X epitope. This activity is unique, as all reported antennary fucosidases are hindered by a sialylation on these epitopes. This fucosidase has immense potential in LC-based glycomics assay for antennary fucose identification. The mechanism of this activity was elucidated relying on structural characterisation of the enzyme. The presence of an open void in the vicinity of the active site allowed for the unhindered accommodation of a α(2-3)-linked Sia residue which was not the case for other previously reported antennary fucosidases. The information obtained from this chapter will go on to aid efforts in genetic engineering of other antennary fucosidases with hope of introducing such a favourable trait. Such approaches of engineering desirable traits into enzymes have repeatedly proven valuable. A relevant example, the incorporation of a predominant transfucosidase activity into the α-1,3/4-L-fucosidase BbAfcB from Bifidobacterium bifidum was achieved by swapping an α-helical loop with the transfucosidase CpAfc2 from Clostridium perfringens [80]. This has important interest in industrial production of human milk oligosaccharides since the desirable activities from a pathogen derived enzyme was incorporated into a probiotic enzyme, thus avoiding possible controversy.

A GlcNAcase was identified that is specific to bisecting GlcNAc residues which gives it a novelty among known GlcNAcases [77]. However, this enzyme was highly limited in its activity as it gets hindered by the presence of galactosylation and sialylation. Chapter 3 is an attempt to understand the mechanism of the enzyme in more detail. Although the mechanism of antennary GlcNAcase catalysis [81] is well known, the mechanism behind its specificity to bisecting GlcNAc residues remains a mystery due to the lack of co-crystallised glycans within its active site. Often these GlcNAcases rely on associated residues encompassing the entry to the active site to regulate the positioning of glycan's antennary arms or bisection [77, 82]. Such previously described regulatory residues [77, 82] could not be identified within this structure. Furthermore, attempts at computer-assisted docking of the glycan into the active site were not conclusive. Follow-up research would be required to understand the regulatory mechanism of the enzyme specificity.

On the importance of interdisciplinary research

Finally, I would like to end this chapter by saying that glycomics like so many other sectors of analytical chemistry, heavily relies on interdisciplinary research. Analytical chemistry projects are heavily dependent on instrumentation and assay development, and thus we require a constant input from other sciences to develop and advance our methods. This brings me to the importance of interdisciplinary collaboration in the research of this thesis as it would not be possible without a consortium-like investigation by analytical chemists, biochemists, structural biologists, and process engineers for commercialisation of potential future products.

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