Glycoconj J (2013) 30:137–146
DOI 10.1007/s10719-012-9443-9

Strategies for the profiling, characterisation and detailed structural analysis of *N*-linked oligosaccharides

Tharmala Tharmalingam · Barbara Adamczyk · Margaret A. Doherty · Louise Royle · Pauline M. Rudd

Received: 14 March 2012 / Revised: 9 August 2012 / Accepted: 13 August 2012 / Published online: 26 August 2012

Abstract Many post-translational modifications, including glycosylation, are pivotal for the structural integrity, location and functional activity of glycoproteins. Sub-populations of proteins that are relocated or functionally changed by such modifications can change resting proteins into active ones, mediating specific effector functions, as in the case of monoclonal antibodies. To ensure safe and efficacious drugs it is essential to employ appropriate robust, quantitative analytical strategies that can (i) perform detailed glycan structural analysis, (ii) characterise specific subsets of glycans to assess known critical features of therapeutic activities (iii) rapidly profile glycan pools for at-line monitoring or high level batch to batch screening. Here we focus on these aspects of glycan analysis, showing how state-of-the-art technologies are required at all stages during the production of recombinant glycotherapeutics. These data can provide insights into processing pathways and suggest markers for intervention at critical control points in bioprocessing and also critical decision points in disease and drug monitoring in patients. Importantly, these tools are now enabling the first glycome/genome studies in large populations, allowing the integration of glycomics into other 'omics platforms in a systems biology context.

Keywords Glycosylation · Glycan analysis · High throughput · Glycoprofiling · Database

T. Tharmalingam · B. Adamczyk · M. A. Doherty · P. M. Rudd (✉)
NIBRT Glycobiology Laboratory, NIBRT - The National Institute for Bioprocessing Research and Training,
Fosters Avenue, Mount Merrion, Blackrock,
Co. Dublin, Ireland
e-mail: pauline.rudd@nibrt.ie

L. Royle
Ludger Ltd., Culham Science Centre,
Oxfordshire OX14 3EB, UK

Abbreviations

2AB	2-aminobenzamide
ADCC	Antibody dependent cell cytotoxicity
APTS	8-aminopyrene-1,3,6-trisulfonate
BEH	Ethylene Bridged Hybrid
CE-LIF	Capillary electrophoresis with laser induced fluorescence
CQA	Critical quality attribute
EMA	European Medicines Agency
EPO	Erythropoietin
ESI-MS	Electrospray ionization – mass spectrometry
FDA	Food and Drug Administration
FLD	Fluorescence detector
FUT6 and FUT8	Fucosyltransferase 6 and 8
GWAS	Genome-wide association study
HILIC	Hydrophilic interaction chromatography
HNF1α	Hepatocyte nuclear factor 1α
HPLC	High performance liquid chromatography
IgG	Immunoglobulin G
LC-MS	Liquid chromatography – mass spectrometry
MALDI	Matrix-assisted laser desorption/ionization
NMR	Nuclear magnetic resonance
PAT	Process analytical technology
PGC	Porous graphitized carbon
RP	Reversed phase
SNPs	Single nucleotide polymorphisms
UPLC	Ultra performance liquid chromatography
WAX	Weak anion exchange

Introduction

Amongst the 400 known post-translational modifications glycosylation is arguably the most abundant and complex. The biosynthetic pathways for glycan processing, although dependent on gene products, are non-template driven and reflect the many and varied parameters, which precede the generation of fully modified glycoproteins. Moreover both cell surface and secreted glycoproteins consist of many sub-populations known as glycoforms that can provide a non-template driven response to a local environment, enabling the relocation of subpopulations of cells or secreted proteins and alterations in their functional activities.

By the same token, sub-populations of therapeutic proteins can also be directed to different locations in the patient, for example non-sialylated Erythropoietin (EPO) is bound to the asialo glycoprotein receptor as it passes through the liver, where it is rapidly (3 min) cleared. In contrast, the population of EPO that is fully sialylated remains in the patient for 3 h. It survives its passage through the liver and reaches the kidneys where it can bind to the EPO receptor and initiate the up regulation of erythrocyte production [1].

The proper control of glycosylation and appropriate analysis at various stages of production is therefore essential for the safety and efficacy of drugs including the delivery of biologically active molecules to the patient. Eliminating non-active drug compounds from the formulation can have a significant impact on costs and more importantly, on the wellbeing of patients. Intervals between drug administrations can be increased and the likelihood of drug resistance through immunological intolerance can be decreased if the patient receives lower levels of protein.

There are three basic levels of glycan analysis. Each provides a particular level of high quality information using robust, repeatable technologies. Each level delivers specific information, some contain more detail than others, but all of the data are high quality and the detail that they provide is designed to answer particular questions. The art is to know which level to select for answering any particular question [2].

Glycoprofiling (fingerprinting, patterning), oligosaccharide characterisation and glycan analysis are often used interchangeably. Although they are inter-related, each strategy provides different kinds of information; therefore in the interests of clarity, it is important that a definition of each term is adopted.

Glycoprofiling involves the one dimensional separation of a glycan pool by a single technique to provide a signature that gives a simple overview or snapshot of a glycan pool and from which preliminary (unconfirmed) information may be surmised. Appropriate technologies that provide different one-dimensional windows on this world include Nuclear Magnetic Resonance (NMR) (structural determination), High Performance Liquid Chromatography (HPLC) (separation by physical parameters such as lipophilicity or charge), Capillary Electrophoresis (CE) (separation of labeled glycans by mass:charge ratio), lectin chips (separation by binding properties), and Mass Spectrometry (MS) (separation by mass). The higher the resolution of the technology the more useful it will be for revealing differences between the profiles that are generated.

Glycan characterisation uses the same technologies to separate glycan pools into different groups or types of glycans. Based on previous experience, the pools can be labeled and data can be interpreted further (though still not unambiguously). Examples include MS separations of di, mono- and non-galactosylated IgG glycans or the weak anion exchange (WAX) separations that separate glycans into neutral, mono-, di-, tri- and tetra sialylated pools. Used after full structural analysis, this approach is a convenient way to highlight defined critical features and can provide relative quantitation of the different glycan pools, depending on the efficiency of the separation and the performance of the technique.

Detailed (full) structural analysis requires extensive determinations of monosaccharide sequence, anomericity and linkage of the majority of the glycans in the pool. In common with good practice in any analytical enterprise, 2-3- and even 5-D orthogonal technologies are required, first to assign preliminary structures and then to confirm the assignments. Full structural analysis also includes absolute or relative quantitation of assigned structures. For example, a WAX separation of EPO glycans into pools of differently charged glycans (1-D) is complemented by HILIC separation of each pool (2-D). The digestion of aliquots of the pool by exoglycosidase arrays (3-D) is then used to determine the sequence, anomericity and linkage of different glycans together with their relative quantitation [1]. Mass spectrometry follows to confirm that assigned structures have compositions that are consistent with composition data (4-D) and any structural ambiguities that remain can be resolved by ESI MS and ion fragmentation (5-D). Once the full analysis has been completed, quantitative data can best be obtained from HILIC analysis of fluorescently labeled glycans by inputting detailed structural information from the other techniques as necessary to resolve ambiguities. Full structural analysis also provides the information needed to design strategies to quantify a particular critical quality attribute (CQA), such as the level of core fucose in antibodies designed to initiate antigen dependent cell cytotoxicity (ADCC), the levels of antigenic α-gal residues or sialyl lewis x epitopes that may be a useful marker to follow inflammation and metastasis.

Enabling technologies

These exacting analytical requirements together with the complementary need for the rapid inexpensive analysis of

biotherapeutics and samples of many thousands of patients in genomic glycomics/studies or in clinical situations have driven the recent dramatic improvements in instrumentation and more economical high throughput (hands-off technologies). Combined with this, increased resolution, miniaturisation and speed are steadily bringing down the costs of glycan profiling, characterisation and detailed analysis. At the same time the generation of data bases and dedicated software have made the interpretation of data from HILIC and other LC separations, CE, and MS more accessible to the less experienced glycoscientist and an invaluable aid to everyone.

To be useful, technologies must be robust, reproducible, have high resolution, produce minimal artefacts, have high signal to noise ratios, be scalable, accurate, precise and based on separation parameters that are linked to structure (predictable). Glycobase, (http://glycobase.nibrt.ie/glycobase/show_nibrt.action) being developed in the NIBRT Dublin-Oxford group is an experimental database in which oligosaccharides have unique glycan identifiers that can be linked to other identifiers (such as different symbolic notations) and to many separation parameters (such HILIC, CE, MS, RP and so on). Standard deviations are included where this is appropriate [3, 4].

HPLC and UPLC

Considerations in glycosylation analysis include the time required for sample preparation, data collection and interpretation of results. There is sometimes a trade-off between resolution and speed. Usually, in a research project data quality takes precedence because few samples are being run and the time taken to interpret the data is a far more significant rate determining step than the separation time. On the other hand, when monitoring the effects of hundreds of media additives or incubation conditions for process analytical technology (PAT), or profiling of thousands of serum glycomes in a genome wide association study (GWAS) speed and miniaturization to reduce time and costs become more and more important.

Classically, HPLC technologies require long run times (3 h) to achieve high resolution of all released oligosaccharides (both *N*- and *O*-) in one profile. A more rapid alternative to HPLC is ultra-performance liquid chromatography (UPLC), which exploits smaller particle size chromatographic packing material and can withstand high pressures, providing greater resolution capability and significantly reduced separation times (30 min or less) [5, 6]. UPLC is fast becoming the separation method of choice because it provides faster and more efficient separations than the HILIC amide columns and is cheaper because everything is miniaturized. This therefore results in faster detection of problems in production and decreased analytical costs in the both the biopharmaceutical industry [6] and in monitoring diseases [7].

The resolution and retention of glycans is based on the affinity of the glycans for the matrix used for separation. After PNGaseF release and labeling (*e.g.* 2AB) glycan pools can be separated by HILIC, WAX, or reversed phase (RP) chromatography. Among these techniques HILIC separation is the technique most commonly used because it is straightforward, relatively cheap and very robust. This technique enables rapid, quantitative, comparative glycan profiling and characterisation across multiple runs and systems. In combination with a dextran hydrolysate ladder to normalize data to glucose units [8, 9]. HILIC is also an invaluable way to approach detailed structural analysis. HILIC separation by HPLC is based on an amide based stationary phase where the polar functional groups interact with the hydroxyl groups of the sugars and by UPLC is based on BEH (Ethylene Bridged Hybrid) stationary phase.

In recent years, instrument and reagent manufacturers have become more cognisant of the emerging market for all types of glycan analysis. This has allowed the field to move forward exponentially. In the past, glycoanalysts used instruments and reagents, such as the TSK amide HPLC column phases, that were originally designed for protein separations but were adapted by glycospecialists for oligosaccharide analysis. A breakthrough came in 2010 with the introduction of BEH columns specifically designed by Waters to exploit the differences in the hydrophilicity of different oligosaccharides [10] and the result is that we can now separate to baseline 24 peaks in the IgG glycan pool [11] compared with 16 on the Amide 80 columns and 3 on the P4 gel filtration medium used previously. An example of the peak resolution is shown in Fig. 1, where the separation of human IgG samples and the glycans associated with the major peaks are shown. The top profile shows the incomplete resolution of structures, where many glycans co-elute. With the introduction of the BEH glycan column, the glycans are resolved into individual peaks.

Recent studies have utilized the UPLC based technology for the *N*-glycan analysis of erythropoietin [13] and to identify cancer associated alterations in the serum of patients [10, 14]. Further developments have included a multiplexed labeling strategy to dramatically increase the sample throughput and provide a more rapid glycomic profiling method [15]. WAX and RP chromatography are very convenient for glycan characterisation: WAX separates glycans into pools depending on their charge and RP separates different classes of glycans such as oligomannose, bisected structures and fucosylated structures from each other [16].

CE-LIF

Capillary electrophoresis with laser induced fluorescence (CE-LIF) is another powerful technique suitable for the profiling of oligosaccharides labeled with APTS (8-aminopyrene-

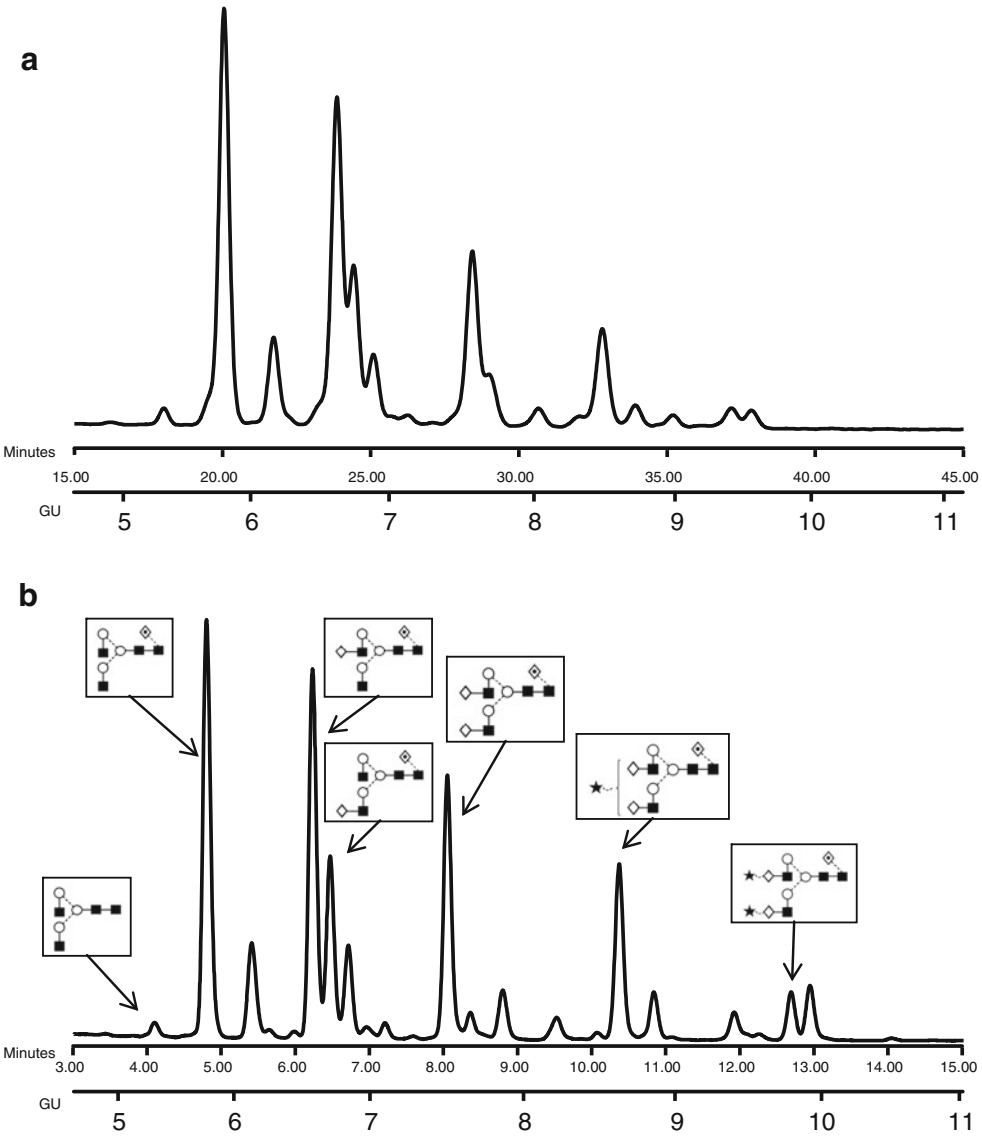

Fig. 1 The separation of human IgG *N*-glycans by HPLC (**a**) and UPLC (**b**). All 36 reported structures were identified; however, selected structures are annotated for the purposes of illustration [12]

1,3,6-Trisulfonate). The high resolution separation is driven by an electric field mediated migration and is therefore based on charge to hydrodynamic volume ratio. Methods for improved sample preparation for CE-LIF is currently being developed [17–19]. Multicapillary formats such as those developed by Ruhaak and colleagues enable high throughput profiling of glycosylation changes [20]. The main problem—peak identification- is being addressed by the development of a CE data base (Dublin-Oxford Glycobase v3.1) using an APTS maltrin ladder to normalize data. This facility now makes CE-LIF a feasible technology for detailed structural analysis. CE-LIF has been compared with UPLC for the comprehensive characterisation of *N*-glycans released from human IgG and, provided the use of an internal standard ladder, the data base and enzyme array digestions, this orthogonal technique is suitable for glycan profiling, characterisation and detailed structural analysis [21]. In common with HPLC, CE-LIF can be coupled with MS, but this remains a challenging enterprise for the novice. Clinical applications of this technology have been described for the detection of chronic liver disease [22].

Mass spectrometry

Mass spectrometry (MS) of oligosaccharides has attracted the major interest of instrument manufactures and there are many instruments and technologies, a description of which is beyond the scope of this review [23]. However, briefly MALDI TOF is a straightforward technique that can be used for rapidly profiling and characterizing neutral glycans and more advanced instruments (ESI QTOF) are able to fragment selected ions and are very powerful for the identification of complicated structures. One particular challenge facing mass spectrometry for detailed structural analysis is the fact that glycans are resolved according to their mass. Many algorithms exist to predict composition from mass, however most combinations

of hexoses, HexNAcs and other monosaccharides exist as structural isomers with the same mass/composition. Although fragmentation can solve some of the issues it is hard to obtain robust quantitative data. Numerous front end separation techniques, such as the coupling of liquid chromatography to mass spectrometry have been developed to address this issue [24–26]. Various chromatographic media such as PGC and C18 have been developed and optimized [27, 28] while columns have been miniaturized to nanoflow levels ensuring improved sensitivity.

Advances in nano technology have led to the development of chip-based analytical tools [29–31]. Although in its early days, the advent of chip-based microfluidics promises even more rapid glycoprofiling strategies for recombinant glycoproteins in the future. Microfluidic chips can be used for profiling and characterisation depending on the separation phases etched into their surfaces. An HPLC-chip device with integrated sample preparation, glycan release, labeling and separation has been developed and was readily demonstrated for a broad range of applications [32]. Glycan analysis with detailed linkage information can be acquired by flowing the eluent from the chip into a mass spectrometry; this however, requires extensive data interpretation. Other powerful developments in mass spectrometry are the coupling of fluorescence detection to mass spectrometry. HILIC UPLC/FLD/QTof MS is a powerful tool for the characterisation of complex glycan samples where the UPLC system is directly interfaced into a QTof MS, and therefore introducing online coupling of MS directly to fluorescence [33]. This has been used in the analysis of the *N*-glycans from a recombinant mAb (Trastuzumab) [34] and coagulation factor IX [33].

Robotic platform for high throughput glycan analysis

All profiling, characterisation and detailed structural analysis with accurate quantitation of glycan structures requires sample preparation. For *N*-glycans this usually involves enzymatic release using PNGase F or PNGase A. This is still time consuming and requires multiple steps. Most steps are optimized for high throughput mode (96 well plate) but manual handling of the samples is often used. Further developments in the speed of sample preparation are essential from a biomarker discovery angle but also importantly in the bioprocessing industry. This requires an integrated system for glycoprofiling to determine batch-to-batch consistency of glycosylation CQA for the recombinant protein.

The current method for sample preparation involves reduction, alkylation, immobilization, PNGase F/A treatment, glycan extraction, concentration, labeling, then clean-up of excess label—a workup that can, if not optimised, take several days [9]. A robotics platform, developed in 2008 [9], based on a 96 well plate format, can be adapted for analysing multiple samples. The methodology has been automated and optimised for increased efficiency and reproducibility of glycan release and labeling. The platform can deliver the glycoprofile of samples, from removal of the glycan from the initial protein to acquisition of the first chromatogram, condensed to as little as 4 h depending on the glycoprotein thereby enhancing the efficiency of the analysis [35].

Developments in enzyme immobilization in high throughput microchromatography units have been described recently [36]. The capacity of running 96 samples in parallel for the enzymatic digestion of glycans is a huge step forward and enables the automation of multiple sample preparation. However, the limitation still exists in the availability of enzymes. The exoglycosidase matrix mediated sequencing of complex glycan pools requires enzymes for the cleavage of specific linkages in order to elucidate structural information regarding complex glycan samples. An example of this is the lack of an enzyme for the specific removal of *N*-glycolylneuraminic acid. Sialidases available currently are capable of removing both *N*-acetyl- and *N*-glycolylneuraminic acid with α2-3, 6, 8 or 9 linkages. As the methods for the separation and resolution of glycans are enhanced, with the increased efficiency of separations, a larger array of enzymes with enhanced specificity is required to ensure the complete identification of glycan structures.

Data analysis

Recent developments in high throughput glycan profiling rapidly generate very large amounts of data. In addition, the complexity and differences between various platforms causes data analysis to be very demanding and time consuming. Interpretation of these results is a challenging task, which necessitates bioinformatic solutions in the forms of databases and new tools to facilitate data processing, analysis and interpretation.

In contrast to the proteomics and genomics fields, glycoscience still requires comprehensive and accessible databases that summarize the structure, characteristics, and potential function of glycans, which have been experimentally verified and reported in the literature [37]. Most of them have their own special focus representing collections of experimental data from HPLC, MS, NMR or microarray experiments [37]. Initiatives to establish cross-links between data acquired from different techniques are beginning to bear fruit.

The development of GlycoBase (http://glycobase.nibrt.ie/glycobase/show_nibrt.action) began in the Oxford Glycobiology Institute in 1996 [8] as an aid to data interpretation. The early versions contained HPLC normalized retention data, expressed as Glucose Units, for 2-AB labeled *N*-glycan structures together with expected products of exoglycosidase digestions. All structures were determined by a combination of HILIC-HPLC with exoglycosidase sequencing and mass

spectrometry (MALDI-MS, ESI-MS, ESI-MS/MS, LC-MS, LC-ESI-MS/MS) [3]. The database is a work in progress and today, the latest version gives information about structure, exoglycosidase array digestions, mass/composition and publication references for the reported structures [4]. The latest version of this database (GlycoBase 3.1) contains GU values for more than 600 2-AB labeled *N*-linked glycan structures by HILIC-UPLC and HILIC-HPLC technologies. In addition, data for CE separations are available and the ongoing expansion of these databases will include data from alternative glycan separation methods such as RP-UPLC and MS composition and fragmentation data. There is also an *O*-link data base and one for milk oligosaccharides.

EuroCarbDB (http://eurocarbdb.org) funded by the European Union gathered experts in the glyco-bioinformatics field to link together rational databases containing analytical data from HPLC, MS and NMR In particular, GlycoWorkbench is a software tool developed by EuroCarbDB for the manual interpretation of MS data, where structures can be proposed by the user by comparison of data against a theoretical list of fragment masses [38]. In addition, the database has been complemented by a set of tools, specifically designed to assist the elucidation and submission of glycan structure and experimental data when used in conjunction with contemporary carbohydrate research workflows [37–40]. The UniCarb-DB project was initiated in 2009 as a continuation of the EuroCarbDB with the ambition to extend the existing database and implement an innovative approach to connect glycomics with systems biology [41].

Applications of the technology

Biomarker discovery

Once glycan structures have been determined, the next challenge is the interpretation of the importance, in terms of biology, of the structures present. Aberrations in glycosylation have proven to be important for the detection of disease. Examples of such diseases can be multiple types of cancers (for a recent review see [42]), rheumatoid arthritis [43], galactosemia [44, 45], and schizophrenia [46, 47].

Controlling glycosylation in biopharmaceutical production

Advancements in the biopharmaceutical industry have led to the new generation of monoclonal antibodies that could be valuable for medicine and industrial use. Their potential as therapeutics has continued to be explored at an exponential rate. Safety and efficacy of these therapeutics have been a major concern for regulatory agencies as alterations in manufacturing may influence glycosylation significantly [48]. In order to ensure the safety of patients and the efficacy of the therapeutic antibodies, drug manufactures must measure and control glycosylation throughout the recombinant protein production. Even small changes in cell culture conditions may cause aberrant glycosylation, which may lead to a number of potential risks [49].

In industrial applications, the biopharmaceutical companies have strict production guidelines for the manufacturing of the recombinant therapeutics, as outlined by the EMA (*European Medicines Agency*) and FDA (*Food and Drug Administration*). These guidelines outline the critical features of glycosylation that would be dangerous for use as therapeutics, and thus the glycosylation must be monitored [50, 51]. An example where non-human glycosylation has caused issues in the past is a monoclonal antibody with glycans containing α-Gal epitopes which led to immunological reactions when administered [52]. Therefore, all regulatory agencies have increased pressure on biomanufacturers to assure satisfactory quality control programmes with special emphasis placed on controlling glycosylation.

Systems biology

The establishment of very robust, repeatable and quantitative HPLC technologies for the high throughput glycoprofiling enable the comparisons large cohorts of samples in short time periods. Following extensive detailed structural analysis of the serum glycome [9] a major study profiling and characterising the human plasma *N*-glycome from 1,008 individuals was carried out. Thirty-three glycan features from each individual were quantified to evaluate the effects of variability, heritability and environmental factors on the levels of groups of plasma glycans [53]. Using the same technology, Pucic *et al.* isolated and analysed IgG from three isolated populations, totalling 2,298 individuals [11].

The generation of glycomic information of the same calibre as genomics or proteomics, has led to collaborative studies such as that published by Lauc *et al.* [54]. This was the first comprehensive GWAS study, which correlated levels of glycans with SNPs, in 2,705 individuals in 3 population cohorts from Croatia and Scotland. Significant associations between glycans and genes were revealed by relating genomics data with glycomics data. The analysis reported an influence of Hepatocyte Nuclear Factor 1A (HNF1A) and fucosyltransferase genes FUT6 and FUT8 on the *N*-glycome from human plasma [54].

Another breakthrough study on the systems biology interface revealed three novel gene associations with variation in human plasma *N*-glycome [15]. This study encompassed both glycomics and genomics where data was obtained from over 3,500 European adults. In addition to previously published polymorphisms (HNF1A, FUT 6, FUT 8) study identified novel associations with B3GAT1, SLC9A9 and MGAT5. It is important to mention that neither B3GAT1 nor SLC9A9

had previously been associated functionally with glycosylation of plasma proteins [15]. This study showed that glycoanalytical technology is now sufficiently developed to join genomics and other major fields in systems biology.

Understanding carbohydrate-protein interactions

Carbohydrate microarray technology is a new development that is revolutionising the study of carbohydrate-protein interactions and the elucidation of their specificities. Protein–glycan and glycan–glycan interactions mediate diverse biological processes in cell communication and immunity. Understanding the functional significance of these interactions is of major interest to the scientific community. Since the introduction of carbohydrate microarrays in 2002 [55], multiple platforms have been developed, tested and most importantly proven to provide essential biological information [56–60].

Glycoarrays are useful tools as a large number of glycans can be assessed simultaneously. They can be used in screening strategies for a wide variety of applications, for instance separating glycans according to their terminal monosaccharides in bioprocessing, detecting specific carbohydrate-binding antibodies in disease diagnosis [61–63] and in carbohydrate-mediated recognition events [64]. These studies have also highlighted the issues that need to be addressed, if the glycoarray technology is to become widely useful and available to the wider scientific community and industry. Particular challenges include the chemical synthesis of oligosaccharide reagents and standards, which is currently not possible in a high throughput mode and is the bottleneck for glycoarray development. Oligosaccharides are difficult to synthesize, derivatise, and analyse, and the development of arrays requires platforms that will be compatible with currently used formats in hospitals and laboratories.

Glycoarrays are really a two-phase platform, one phase is the generation of the glycan library, utilised as probes, which are arrayed on the chip [65, 66] and the other is the method of attachment of the glycan library [57, 67], which needs to replicate faithfully the multivalent display of sugars, imitating the presentation of carbohydrates in nature. The conventionally used strategy in the preparation of carbohydrate microarrays is the covalent attachment of chemically modified glycans to custom derivatized surfaces [60]. The most popular solid surfaces for carbohydrate deposition are functionalised glass slides, gold surfaces, microtiter plates and nanoparticles [66, 68, 58]. The microtiter plate platform (ELISA type) is one of the most commonly used formats for studying protein-glycan interactions. It is widely used in the clinic, therefore has the potential to become the first glycoarray platform for true diagnostic applications. Many diagnostic applications of glycoarrays are already emerging in infection and cancer so the future is promising. The next step forward will be the extensive testing in wider population.

Concluding remarks

In this article, current analytical technologies that are able to profile, characterise and provide detailed structural analysis of the glycosylation of natural and recombinant glycoproteins have been briefly described. The advancements in this challenging field have been a significant benefit to the pharmaceutical community in the production of glycosylated therapeutics. In basic science they have opened up exciting possibilities for integrating glycomics with other -omics fields, moving closer to the realisation of a systems glycobiology view of the world taking its place within mainstream science.

Funding TT acknowledges funding from Science Foundation Ireland (Reproductive Biology Research Cluster (RBRC)) [grant number 07/SRC/B1156], BA acknowledges funding from the European Union Seventh Framework Programme (FP7/2007-2013) under grant agreement 215536 (EuroGlycoArrays), MAD acknowledges funding from the European Union Seventh Framework Programme (FP7/2007-2013) under grant agreement 259869 (GlycoBioM). The authors thank Prof. Johannis P. Kamerling for fruitful discussions.

References

1. Shahrokh, Z., Royle, L., Saldova, R., Bones, J., Abrahams, J.L., Artemenko, N.V., Flatman, S., Davies, M., Baycroft, A., Sehgal, S., Heartlein, M.W., Harvey, D.J., Rudd, P.M.: Erythropoietin produced in a human cell line (Dynepo) has significant differences in glycosylation compared with erythropoietins produced in CHO cell lines. Mol. Pharm. **8**(1), 286–296 (2010). doi:10.1021/mp100353a
2. Marino, K., Bones, J., Kattla, J.J., Rudd, P.M.: A systematic approach to protein glycosylation analysis: a path through the maze. Nat. Chem. Biol. **6**(10), 713–723 (2010)
3. Campbell, M.P., Royle, L., Radcliffe, C.M., Dwek, R.A., Rudd, P.M.: GlycoBase and autoGU: tools for HPLC-based glycan analysis. Bioinformatics **24**(9), 1214–1216 (2008). doi:10.1093/bioinformatics/btn090
4. Hilliard, M., Struwe, W., Carta, G., O'Rourke, J., McLoughlin, N., Rudd, P.M., Qing, Y.: A systematic approach to glycan analysis using HILIC-UPLC and an online database of standardized values. In: (2012). http://www.waters.com/waters/library.htm?locale=en_US&lid=134658513
5. Guillarme, D., Ruta, J., Rudaz, S., Veuthey, J.L.: New trends in fast and high-resolution liquid chromatography: a critical comparison of existing approaches. Anal. Bioanal. Chem. **397**(3), 1069–1082 (2010). doi:10.1007/s00216-009-3305-8
6. Novakova, L., Matysova, L., Solich, P.: Advantages of application of UPLC in pharmaceutical analysis. Talanta **68**(3), 908–918 (2006). doi:10.1016/j.talanta.2005.06.035
7. Lame, M.E., Chambers, E.E., Blatnik, M.: Quantitation of amyloid beta peptides Abeta(1–38), Abeta(1–40), and Abeta(1–42) in human cerebrospinal fluid by ultra-performance liquid chromatography-tandem mass spectrometry. Anal. Biochem. **419**(2), 133–139 (2011). doi:10.1016/j.ab.2011.08.010
8. Guile, G.R., Rudd, P.M., Wing, D.R., Prime, S.B., Dwek, R.A.: A rapid high-resolution high-performance liquid chromatographic

method for separating glycan mixtures and analyzing oligosaccharide profiles. Anal. Biochem. **240**(2), 210–226 (1996)
9. Royle, L., Campbell, M.P., Radcliffe, C.M., White, D.M., Harvey, D.J., Abrahams, J.L., Kim, Y.-G., Henry, G.W., Shadick, N.A., Weinblatt, M.E., Lee, D.M., Rudd, P.M., Dwek, R.A.: HPLC-based analysis of serum N-glycans on a 96-well plate platform with dedicated database software. Anal. Biochem. **376**(1), 1–12 (2008)
10. Ahn, J., Bones, J., Yu, Y.Q., Rudd, P.M., Gilar, M.: Separation of 2-aminobenzamide labeled glycans using hydrophilic interaction chromatography columns packed with 1.7um sorbent. J Chromatogr B **878**(3:4), 403–408 (2010)
11. Pucic, M., Knezevic, A., Vidic, J., Adamczyk, B., Novokmet, M., Polasek, O., Gornik, O., Supraha-Goreta, S., Wormald, M.R., Redzic, I., Campbell, H., Wright, A., Hastie, N.D., Wilson, J.F., Rudan, I., Wuhrer, M., Rudd, P.M., Josic, D., Lauc, G.: High throughput isolation and glycosylation analysis of iggâ €"Variability and heritability of the IgG glycome in three isolated human populations". Mol. Cell Proteomics **10**(10), (2011). doi:10.1074/mcp.M111.010090
12. Wormald, M.R., Rudd, P.M., Harvey, D.J., Chang, S.C., Scragg, I.G., Dwek, R.A.: Variations in oligosaccharide-protein interactions in immunoglobulin G determine the site-specific glycosylation profiles and modulate the dynamic moon of the Fc oligosaccharides. Biochemistry **36**(6), 1370–1380 (1997)
13. Bones, J., McLoughlin, N., Hilliard, M., Wynne, K., Karger, B.L., Rudd, P.M.: 2D-LC analysis of BRP 3 erythropoietin N-glycosylation using anion exchange fractionation and hydrophilic interaction UPLC reveals long poly-N-acetyl lactosamine extensions. Anal. Chem. **83**(11), 4154–4162 (2011). doi:10.1021/ac200406z
14. Bones, J., Mittermayr, S., O'Donoghue, N., Guttman, A., Rudd, P.M.: Ultra performance liquid chromatographic profiling of serum N-glycans for fast and efficient identification of cancer associated alterations in glycosylation. Anal. Chem. **82**(24), 10208–10215 (2010). doi:10.1021/ac102860w
15. Huffman, J.E., Knezevic, A., Vitart, V., Kattla, J., Adamczyk, B., Novokmet, M., Igl, W., Pucic, M., Zgaga, L., Johannson, A., Redzic, I., Gornik, O., Zemunik, T., Polasek, O., Kolcic, I., Pehlic, M., Koeleman, C.A.M., Campbell, S., Wild, S.H., Hastie, N.D., Campbell, H., Gyllensten, U., Wuhrer, M., Wilson, J.F., Hayward, C., Rudan, I., Rudd, P.M., Wright, A.F., Lauc, G.: Polymorphisms in B3GAT1, SLC9A9 and MGAT5 are associated with variation within the human plasma N-glycome of 3533 European adults. Hum. Mol. Genet. **20**(24), 5000–5011 (2011). doi:10.1093/hmg/ddr414
16. Domann, P.J., Pardos-Pardos, A.C., Fernandes, D.L., Spencer, D.I.R., Radcliffe, C.M., Royle, L., Dwek, R.A., Rudd, P.M.: Separation-based glycoprofiling approaches using fluorescent labels. Proteomics **7**(S1), 70–76 (2007). doi:10.1002/pmic.200700640
17. Szabo, Z., Guttman, A., Karger, B.L.: Rapid release of N-linked glycans from glycoproteins by pressure-cycling technology. Anal. Chem. **82**(6), 2588–2593 (2010). doi:10.1021/ac100098e
18. Olajos, M., Hajos, P., Bonn, G.K., Guttman, A.: Sample preparation for the analysis of complex carbohydrates by multicapillary gel electrophoresis with light-emitting diode induced fluorescence detection. Anal. Chem. **80**(11), 4241–4246 (2008). doi:10.1021/ac8002598
19. Szabo, Z., Guttman, A., Rejtar, T., Karger, B.L.: Improved sample preparation method for glycan analysis of glycoproteins by CE-LIF and CE-MS. Electrophoresis **31**(8), 1389–1395 (2010). doi:10.1002/elps.201000037
20. Ruhaak, L.R., Hennig, R., Huhn, C., Borowiak, M., Dolhain, R.J., Deelder, A.M., Rapp, E., Wuhrer, M.: Optimized workflow for preparation of APTS-labeled N-glycans allowing high-throughput analysis of human plasma glycomes using 48-channel multiplexed CGE-LIF. J. Proteome Res. **9**(12), 6655–6664 (2010). doi:10.1021/pr100802f
21. Mittermayr, S., Bones, J., Doherty, M., Guttman, A., Rudd, P.M.: Multiplexed analytical glycomics: rapid and confident IgG N-glycan structural elucidation. J. Proteome Res. **10**(8), 3820–3829 (2011). doi:10.1021/pr200371s
22. Vanderschaeghe, D., Szekrenyes, A., Wenz, C., Gassmann, M., Naik, N., Bynum, M., Yin, H., Delanghe, J., Guttman, A., Callewaert, N.: High-throughput profiling of the serum N-glycome on capillary electrophoresis microfluidics systems: toward clinical implementation of GlycoHepatoTest. Anal. Chem. **82**(17), 7408–7415 (2010). doi:10.1021/ac101560a
23. North, S.J., Huang, H.-H., Sundaram, S., Jang-Lee, J., Etienne, A.T., Trollope, A., Al-Chalabi, S., Dell, A., Stanley, P., Haslam, S.M.: Glycomics profiling of Chinese hamster ovary (CHO) cell glycosylation mutants reveals N-glycans of a novel size and complexity. J. Biol. Chem. (2009). doi:10.1074/jbc.M109.068353
24. Hua, S., Lebrilla, C., An, H.J.: Application of nano-LC-based glycomics towards biomarker discovery. Bioanalysis **3**(22), 2573–2585 (2011). doi:10.4155/bio.11.263
25. Kirsch, S., Bindila, L.: Nano-LC and HPLC-chip-ESI-MS: an emerging technique for glycobioanalysis. Bioanalysis **1**(7), 1307–1327 (2009). doi:10.4155/bio.09.110
26. Kosicek, M., Kirsch, S., Bene, R., Trkanjec, Z., Titlic, M., Bindila, L., Peter-Katalinic, J., Hecimovic, S.: Nano-HPLC-MS analysis of phospholipids in cerebrospinal fluid of Alzheimer's disease patients–a pilot study. Anal. Bioanal. Chem. **398**(7–8), 2929–2937 (2010). doi:10.1007/s00216-010-4273-8
27. Ruhaak, L.R., Deelder, A.M., Wuhrer, M.: Oligosaccharide analysis by graphitized carbon liquid chromatography-mass spectrometry. Anal. Bioanal. Chem. **394**(1), 163–174 (2009). doi:10.1007/s00216-009-2664-5
28. Wohlgemuth, J., Karas, M., Jiang, W., Hendriks, R., Andrecht, S.: Enhanced glyco-profiling by specific glycopeptide enrichment and complementary monolithic nano-LC (ZIC-HILIC/RP18e)/ESI-MS analysis. J. Sep. Sci. **33**(6–7), 880–890 (2010). doi:10.1002/jssc.200900771
29. Zamfir, A., Vakhrushev, S., Sterling, A., Niebel, H.J., Allen, M., Peter-Katalinic, J.: Fully automated chip-based mass spectrometry for complex carbohydrate system analysis. Anal. Chem. **76**(7), 2046–2054 (2004). doi:10.1021/ac035320q
30. Zamfir, A.D., Bindila, L., Lion, N., Allen, M., Girault, H.H., Peter-Katalinic, J.: Chip electrospray mass spectrometry for carbohydrate analysis. Electrophoresis **26**(19), 3650–3673 (2005). doi:10.1002/elps.200500101
31. Hua, S., An, H.J., Ozcan, S., Ro, G.S., Soares, S., DeVere-White, R., Lebrilla, C.B.: Comprehensive native glycan profiling with isomer separation and quantitation for the discovery of cancer biomarkers. Analyst **136**(18), 3663–3671 (2011). doi:10.1039/c1an15093f
32. Bynum, M.A., Yin, H., Felts, K., Lee, Y.M., Monell, C.R., Killeen, K.: Characterization of IgG N-glycans employing a microfluidic chip that integrates glycan cleavage, sample purification, LC separation, and MS detection. Anal. Chem. **81**(21), 8818–8825 (2009). doi:10.1021/ac901326u
33. Yu, Y.Q.: Analysis of N-linked glycans from coagulation factor IX, recombinant and plasma derived, using HILIC UPLC/FLR/QTof MS. In: Waters (ed.) Application Note, (2011). http://www.waters.com/waters/library.htm?locale=en_US&lid=10196714&cid=511436
34. Yu, Y.Q., Ahn, J., Gilar, M.: Trastuzumab glycan batch-to-batch profiling using a UPLC/FLR/MS platform In: Waters (ed.) Application Note, (2011). http://www.waters.com/waters/library.htm?locale=en_US&lid=10166999&cid=511436
35. Kattla, J.J., Struwe, W.B., Campbell, M.P., Doherty, M., Adamczyk, B., Saldova, R., Rudd, P.M.: Protein glycosylation. In: Moo-Young, M. (ed.) Comprehensive Biotechnology, vol. 3. pp. 467–486.

Elsevier (2011). http://www.sciencedirect.com/science/referenceworks/9780080885049
36. Fulton, S., Murphy, S., Reich, J., Van Den Heuvel, Z., Sakowski, R., Smith, R., Agee, S.: A high-throughput microchromatography platform for quantitative analytical scale protein sample preparation. J. Assoc. Lab. Autom. **16**(6), 457–467 (2011)
37. von der Lieth, C.-W., Freire, A.A., Blank, D., Campbell, M.P., Ceroni, A., Damerell, D.R., Dell, A., Dwek, R.A., Ernst, B., Fogh, R., Frank, M., Geyer, H., Geyer, R., Harrison, M.J., Henrick, K., Herget, S., Hull, W.E., Ionides, J., Joshi, H.J., Kamerling, J.P., Leeflang, B.R., Lutteke, T., Lundborg, M., Maass, K., Merry, A., Ranzinger, R., Rosen, J., Royle, L., Rudd, P.M., Schloissnig, S., Stenutz, R., Vranken, W.F., Widmalm, G., Haslam, S.M.: EUROCarbDB: an open-access platform for glycoinformatics. Glycobiology **21**(4), 493–502 (2011). doi:10.1093/glycob/cwq188
38. Ceroni, A., Maass, K., Geyer, H., Geyer, R., Dell, A., Haslam, S.M.: GlycoWorkbench: a tool for the computer-assisted annotation of mass spectra of glycans. J. Proteome Res. **7**(4), 1650–1659 (2008). doi:10.1021/pr7008252
39. Maass, K., Ranzinger, R., Geyer, H., von der Lieth, C.-W., Geyer, R.: "Glyco-peakfinder" – *de novo* composition analysis of glycoconjugates. Proteomics **7**(24), 4435–4444 (2007). doi:10.1002/pmic.200700253
40. Jansson, P.-E., Stenutz, R., Widmalm, G.R.: Sequence determination of oligosaccharides and regular polysaccharides using NMR spectroscopy and a novel Web-based version of the computer program casper. Carbohydr. Res. **341**(8), 1003–1010 (2006)
41. Campbell, M.P., Hayes, C.A., Struwe, W.B., Wilkins, M.R., Aoki-Kinoshita, K.F., Harvey, D.J., Rudd, P.M., Kolarich, D., Lisacek, F., Karlsson, N.G., Packer, N.H.: UniCarbKB: putting the pieces together for glycomics research. Proteomics **11**(21), 4117–4121 (2011). doi:10.1002/pmic.201100302
42. Adamczyk, B., Tharmalingam, T., Rudd, P.M.: Glycans as cancer biomarkers. Biochim. Biophys. Acta (BBA) Gen. Subj. (0), (2012). http://dx.doi.org/10.1016/j.bbagen.2011.12.001. Accessed 9 Dec 2011
43. Ercan, A., Cui, J., Chatterton, D.E.W., Deane, K.D., Hazen, M.M., Brintnell, W., O'Donnell, C.I., Derber, L.A., Weinblatt, M.E., Shadick, N.A., Bell, D.A., Cairns, E., Solomon, D.H., Holers, V.M., Rudd, P.M., Lee, D.M.: Aberrant IgG galactosylation precedes disease onset, correlates with disease activity, and is prevalent in autoantibodies in rheumatoid arthritis. Arthritis Rheum. **62**(8), 2239–2248 (2010). doi:10.1002/art.27533
44. Coman, D.J., Murray, D.W., Byrne, J.C., Rudd, P.M., Bagaglia, P.M., Doran, P.D., Treacy, E.P.: Galactosemia, a single gene disorder with epigenetic consequences. Pediatr. Res. **67**(3), 286–292 (2010)
45. Coss, K.P., Byrne, J.C., Coman, D.J., Adamczyk, B., Abrahams, J.L., Saldova, R., Brown, A.Y., Walsh, O., Hendroff, U., Carolan, C., Rudd, P.M., Treacy, E.P.: IgG N-glycans as potential biomarkers for determining galactose tolerance in Classical Galactosaemia. Mol. Genet. Metab. **105**(2), 212–220 (2012)
46. Stanta, J.L., Saldova, R., Struwe, W.B., Byrne, J.C., Leweke, F.M., Rothermund, M., Rahmoune, H., Levin, Y., Guest, P.C., Bahn, S., Rudd, P.M.: Identification of N-Glycosylation changes in the CSF and serum in patients with schizophrenia. J. Proteome Res. **9**(9), 4476–4489 (2010). doi:10.1021/pr1002356
47. Telford, J.E., Bones, J., McManus, C., Saldova, R., Manning, G., Doherty, M., Leweke, F.M., Rothermundt, M., Guest, P.C., Rahmoune, H.: Anti-psychotic treatment of acute paranoid schizophrenia patients with olanzapine results in altered glycosylation of serum glycoproteins. J Proteome Res. (2012) 11(7):3743–52. doi:10.1021/pr300218h. Accessed 29 May 2012
48. Cosgrave, E.F., Struwe, W.B., Kattla, J.J., Campbell, M.P., Wormald, M.R., Rudd, P.M.: Systems biology glycomics. In: Moo-Young, M. (ed.) Comprehensive Biotehcnology, vol. 1. pp. 427–446. Elsevier (2011). http://www.sciencedirect.com/science/referenceworks/9780080885049
49. Fernandes, D.: Reducing risk in biopharmaceutical production by controlling Glycosylation. Eur. Biopharm. Rev. 92–97 (2004)
50. Covic, A., Cannata-Andia, J., Cancarini, G., Coppo, R., Frazao, J.M., Goldsmith, D., Ronco, P., Spasovski, G.B., Stenvinkel, P., Utas, C., Wiecek, A., Zoccali, C., London, G.: Biosimilars and biopharmaceuticals: what the nephrologists need to know - a position paper by the ERA-EDTA Council. Nephrol. Dial. Transplant. **23**(12), 3731–3737 (2008). doi:10.1093/ndt/gfn519
51. Uçaktürk, E.: Analysis of glycoforms on the glycosylation site and the glycans in monoclonal antibody biopharmaceuticals. J. Sep. Sci. **35**(3), 341–350 (2012). doi:10.1002/jssc.201100684
52. Chung, C.H., Mirakhur, B., Chan, E., Le, Q.-T., Berlin, J., Morse, M., Murphy, B.A., Satinover, S.M., Hosen, J., Mauro, D., Slebos, R.J., Zhou, Q., Gold, D., Hatley, T., Hicklin, D.J., Platts-Mills, T.A.E.: Cetuximab-induced anaphylaxis and IgE specific for galactose-Î±-1,3-Galactose. N. Engl. J. Med. **358**(11), 1109–1117 (2008). doi:10.1056/NEJMoa074943
53. Knezevic, A., Polasek, O., Gornik, O., Rudan, I., Campbell, H., Hayward, C., Wright, A., Kolcic, I., O'Donoghue, N., Bones, J., Rudd, P.M., Lauc, G.: Variability, heritability and environmental determinants of human plasma N-glycome. J. Proteome Res. **8**(2), 694–701 (2008). doi:10.1021/pr800737u
54. Lauc, G., Essafi, A., Huffman, J.E., Hayward, C., Knezevic, A., Kattla, J.J., Polasek, O., Gornik, O., Vitart, V., Abrahams, J.L., Pucic, M., Novokmet, M., Redzic, I., Campbell, S., Wild, S.H., Borovecki, F., Wang, W., Kolcic, I., Zgaga, L., Gyllensten, U., Wilson, J.F., Wright, A.F., Hastie, N.D., Campbell, H., Rudd, P.M., Rudan, I.: Genomics meets glycomics the first GWAS study of human N-glycome identifies HNF1A as a master regulator of plasma protein Fucosylation. PLoS Genet. **6**(12), e1001256 (2010)
55. Fazio, F., Bryan, M.C., Blixt, O., Paulson, J.C., Wong, C.-H.: Synthesis of sugar arrays in microtiter plate. J. Am. Chem. Soc. **124**(48), 14397–14402 (2002). doi:10.1021/ja020887u
56. Horlacher, T., Seeberger, P.H.: Carbohydrate arrays as tools for research and diagnostics. Chem. Soc. Rev. **37**(7), (2008). doi:10.1039/B708016F
57. Disney, M.D., Seeberger, P.H.: The use of carbohydrate microarrays to study carbohydrate-cell interactions and to detect pathogens. Chem. Biol. **11**(12), 1701–1707 (2004)
58. Laurent, N., Voglmeir, J., Flitsch, S.L.: Glycoarrays-tools for determining protein-carbohydrate interactions and glycoenzyme specificity. Chem. Commun. (37), 4400–4412 (2008). doi:10.1039/B806983M
59. Feizi, T., Chai, W.: Oligosaccharide microarrays to decipher the glyco code. Nat. Rev. Mol. Cell Biol. **5**(7), 582–588 (2004)
60. Paulson, J.C., Blixt, O., Collins, B.E.: Sweet spots in functional glycomics. Nat. Chem. Biol. **2**(5), 238–248 (2006)
61. Ratner, D.M., Seeberger, P.H.: Carbohydrate microarrays as tools in HIV glycobiology. Curr. Pharm. Des. **13**(2), 173–183 (2007)
62. Pedersen, J.W., Blixt, O., Bennett, E.P., Tarp, M.A., Dar, I., Mandel, U., Poulsen, S.S., Pedersen, A.E., Rasmussen, S., Jess, P., Clausen, H., Wandall, H.H.: Seromic profiling of colorectal cancer patients with novel glycopeptide microarray. Int. J. Cancer **128**(8), 1860–1871 (2011). doi:10.1002/ijc.25778
63. Lawrie, C.H., Marafioti, T., Hatton, C.S., Dirnhofer, S., Roncador, G., Went, P., Tzankov, A., Pileri, S.A., Pulford, K., Banham, A.H.: Cancer-associated carbohydrate identification in Hodgkin's lymphoma by carbohydrate array profiling. Int. J. Cancer **118**(12), 3161–3166 (2006). doi:10.1002/ijc.21762

64. Park, S., Lee, M.R., Shin, I.: Carbohydrate microarrays as powerful tools in studies of carbohydrate-mediated biological processes. Chem. Commun. (Camb.) (37), 4389–4399 (2008). doi:10.1039/b806699j
65. Hanson, S., Best, M., Bryan, M.C., Wong, C.H.: Chemoenzymatic synthesis of oligosaccharides and glycoproteins. Trends Biochem. Sci. **29**(12), 656–663 (2004). doi:10.1016/j.tibs.2004.10.004
66. Seeberger, P.H., Werz, D.B.: Automated synthesis of oligosaccharides as a basis for drug discovery. Nat. Rev. Drug Discov. **4**(9), 751–763 (2005). doi:10.1038/nrd1823
67. Feizi, T., Chai, W.: Oligosaccharide microarrays to decipher the glyco code. Nat. Rev. Mol. Cell Biol. **5**(7), 582–588 (2004). doi:10.1038/nrm1428
68. Blixt, O., Head, S., Mondala, T., Scanlan, C., Huflejt, M.E., Alvarez, R., Bryan, M.C., Fazio, F., Calarese, D., Stevens, J., Razi, N., Stevens, D.J., Skehel, J.J., van Die, I., Burton, D.R., Wilson, I.A., Cummings, R., Bovin, N., Wong, C.-H., Paulson, J.C.: Printed covalent glycan array for ligand profiling of diverse glycan binding proteins. Proc. Natl. Acad. Sci. U. S. A. **101**(49), 17033–17038 (2004). doi:10.1073/pnas.0407902101