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TUTORIAL REVIEW

Glycan microarrays: new angles and new strategies

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Carbohydrate microarrays, comprising hundreds to thousands of different glycan structures on solid surfaces in a spatially discrete pattern, are sensitive and versatile tools for the analysis of glycosylation changes in complex biological samples. Glycoarrays are also suitable for monitoring multiple molecular interactions with biomolecules where sugars are involved, offering a large variety of bioassay options. In this paper we review the most important glycan microarray types currently used with their main applications, and discuss some of the future challenges the technology faces.

1 Introduction

The surface of virtually every living cell is decorated with highly diverse glycan structures (glycoproteins, proteoglycans, glycosaminoglycans, *etc.*) that mediate a plethora of biological events.[1] The emerging field of glycobiology is devoted to defining the structure–function relationship of glycans in numerous clinically significant biological processes, including, viral and bacterial infection, tumorigenesis, the immune response and many receptor-mediated signaling processes.[2] Specific glycosylation patterns may serve as markers for the early stages of diseases or malignancy prior to metastasis.[3] Glycan microarrays represent a versatile toolset and provide the analytical means for a large variety of such biomedical applications.

Glycoarray development started at the turn of the 21st century, due to the critical need for global and high-throughput methods for the systematic deciphering of glycosylation patterns and identification of the specificities of glycan binding proteins in order to enable detailed investigations of their biological significance as well as their roles in various diseases.[4] After a few years, following the first reports on the use of glycan microarrays, there was an outburst of interest in the generation of glycan libraries, the development of efficient glycan immobilization methods on array surfaces, and their application in the analysis of glycan binding proteins and other biomolecules.[5] For the time being, glycan microarray applications are mainly focused on the analysis of microorganism and mammalian glycans.[6] Since the comprehensive characterization of complex glycan structures is a very demanding task, it has taken many years for the field to develop the appropriate methods for glycoarray technology.[7] Currently, one can utilize the benefits of that work by having access to structurally defined, chemo/enzymatically synthesized glycans, which can be used in an array format to shed light on functional recognition and other important biochemical features that involve glycosylation changes. The use of natural glycan arrays is another strategy, which employs intact glycans derived from natural sources.[8,9]

Microarray and microchip technologies have been successfully applied in genomics for decades. Protein and glycan microarrays, on the other hand, share a common handicap as the required capture molecules are not readily available and their production, especially of glycan targets, is time consuming and labor intensive. Finally, the amplification techniques commonly used in nucleic acid analysis cannot be used in proteomics and glycomics, therefore in most instances one must work with limited amounts of samples, requiring detection methods with higher sensitivity and capture molecules with greater specificity.[10] This review gives a quick overview of the glycoarray methods currently used and the latest developments in glycan microarray technology along with its major analytical applications.

2 Brief overview of glycan microarray fabrication methods

2.1 Contact and non-contact printing

Progress in microarray technology has provided the necessary instrumentation in the form of arraying robots and printers that can also be used to produce glycan microarrays.[9] Scanning devices, necessary for monitoring detection signals from binding assays, are readily accessible as well. Glycan microarray printing can generally be categorized into contact printing and non-contact printing methods. In contact printing, a set of steel pins (up to 48) are immersed into functionalized glycan solutions in a multi-well source plate, and transferred by directly touching the surface of the wafer slides. The amount of solution delivered to the wafer is a function of the time the pin is in contact with the surface. Depending on the pin type, the samples are usually pre-blotted on a test surface to assure consistent spot morphology before the actual microarray is printed. The amount of pre-blotting and contact time can be

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tuned so that ~0.5 nL per spot is printed rapidly and reproducibly.[11]

Non-contact printing, in most instances is accomplished by means of a piezoelectric printer that controls the delivery of the reagent solution at a level of ~0.3 nL (with <5% intra-tip variation) *via* a glass capillary using highly controlled electrical impulses.[12] This process can be fine-tuned by optimizing the printing buffer composition for uniform delivery from each tip (<10% inter-tip variation) to assure more precise printing, relative to contact printing. The size and morphology of the printed spots are relatively homogeneous even without contacting the substrate, resulting in more precise readouts than those obtained with contact printing.

The accuracy of printing by either approach is especially important when quantitative or semi-quantitative studies are required.[13] Large arrays with thousands of spots or even multiple sub-arrays on a single slide can be obtained by means of non-contact printing, this permits multiple analyses to be carried out on various sections of the slide.

2.2 Covalent and non-covalent binding

In recent years a large number of glycan based microarray platforms, based on specific (covalent or non-covalent) immobilization of chemically conjugated glycans on modified surfaces, have been developed for high-throughput glycomic studies.[14] The actual problem in hand is to immobilize the spotted glycan probes on the slides to produce stable microarrays. Nitrocellulose coated glass slides are the most common solid surface wafers for non-covalent attachment.[15] Covalent immobilization, on the other hand, provides more specific attachment of the glycan probes through known functional groups and orientation.[16] For example, Park *et al.*[17] have synthesized maleimide-derivatized carbohydrates and have spotted them onto a thiol-modified glass surface as shown in Fig. 1. Ratner *et al.*[8] utilized the opposite approach by reacting thiol-derivatized carbohydrates with maleimide modified surfaces. The Diels–Alder reaction was applied by Houseman and Mrksich[18] between cyclopentadiene-derivatized carbohydrates and a benzoquinone-functionalized surface. Recently, Mercey *et al.*[19] immobilized pyrrole-derivatized oligosaccharides on modified surfaces by electro-copolymerization. Other functionalized carbohydrates, such as those derivatized with amine-, aldehyde-, hydrazide-, azide-groups have also been used to construct carbohydrate microarrays.[20]

Most arrays are produced by printing monovalent carbohydrates onto a wafer surface. One of the strategies for modulating probe density is to vary the concentration of the monovalent carbohydrates in the print solution.[22] With this approach, however, the array surface acts as a multivalent scaffold. An alternative strategy is first to produce multivalent glycans with varying density, and then to print the conjugates onto a solid surface to generate an array with different multivalent components.[23]

In recent years, glycan microarrays have evolved towards more advanced formats. Sugar structures have been directly synthesized on bead surfaces, immobilized on silica wafers or in ELISA plate wells.[24] The advent of high precision robotic arraying systems and high resolution imaging has also transformed the field by permitting substantial miniaturization, *i.e.*, tens of thousands of carbohydrates or other array features can be affixed and imaged on a standard size microscope slide. These microarrays permit high-throughput analysis of many potential combinations of structures, while using only minuscule amounts of each of the carbohydrates as probes.[25]

2.3 Chemo/enzymatic synthesis

Glycan microarrays have been very successful in screening glycan binding proteins to provide valuable information on their specificity and binding properties. Most arrays are based on glycan probes obtained by chemical synthesis and/or enzymatic methods. Thus, the expansion of such microarrays is limited due to the inherent complexity of glycan structures and difficulties in their synthesis. Natural glycan arrays are good alternatives to further functional glycomics research.[26]

In the early days, libraries of glycans isolated from natural sources were used to produce glycan microarray, and this class of carbohydrates continues to be featured as components of diverse glycan libraries. The synthesis of glycans by chemical or chemo/enzymatic methods presents a viable alternative for the isolation of natural glycans. But unfortunately, there are still no systematic methods for the routine synthesis of glycans of defined sequence like those available for DNA and proteins.[1]

Bohorov *et al.*[27] developed a glycan derivatization method using a modified hydroxylamine that retained a closed-ring form at the reducing end. However, the lack of spectroscopic properties of the linker limited its application in natural glycan array development. Microscale derivatization, characterization, and purification were essential due to the limited amounts of glycans available. This strategy preserved the structural glycan features required for antibody recognition, and allowed the development of natural arrays of fluorescent glycans in which the cyclic pyranose structure of the reducing-end sugar residue was retained.[27,28] Although microscale derivatization of individual free glycans offered a rapid route for building a library, the ability to derivatize a glycan mixture from natural sources and to separate them was more attractive.[29]

A novel method has been reported for the generation of quantifiable glycan libraries by combining the protease

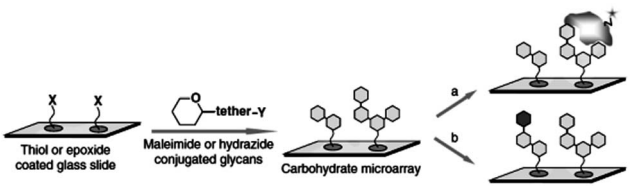

Fig. 1 Fabrication of glycan microarrays by respective immobilization of maleimide or hydrazide-conjugated glycans onto thiol or epoxide derivatized glass slides and their applications in (a) the determination of the binding affinities between glycans and proteins and (b) profiling of glycosyltransferase activities. Reproduced with permission from Ref. 21.

digestion of glycoproteins and Fmoc chemistry.[4] Glycans were derived by enzymatic or chemical cleavage from natural sources, such as glycoproteins and glycolipids, followed by reacting them with a fluorescent linker, and purification by multidimensional chromatography to obtain tagged glycan libraries (TGLs).[30] The purified tagged glycans were printed as glycan microarrays. Fig. 2 shows the use of such an oligosaccharide microarray system.[31,32]

3 Glycoarray applications

Functional glycomics has attracted great interest due to discoveries about the important roles of complex glycoconjugates in biological and pathological processes.[33] Glycan microarrays, in which glycans are immobilized onto activated wafer surfaces and then ~~treated~~ with the sample of interest (*e.g.*, cell lysates, proteins, pathogens, *etc.*), have been shown to be successful in functional glycomics studies.[26] Gene and protein microarray technologies have transformed the investigation of protein–glycan interactions into microscale based assays capable of simultaneously analyzing hundreds to thousands of glycans for their binding ability. This method can be used to test the binding specificities of potential glycan binding proteins. Detection of the interaction is usually accomplished by fluorescent methods, using lectins, antibodies, viruses and receptors.[34] In addition, microarray technology enables large numbers of samples to be analyzed and can be used in various biosensing assays with appropriate imaging methods, *e.g.*, surface plasmon resonance.[35] Glycoarrays hold the promise for an individualized and vastly improved standard for healthcare, which will have great impact on the way clinical diagnostics will be practiced in the future. Printed glycan arrays (PGA) are one of the newest of such high-throughput microarray technologies, allowing the automated detection of an unlimited number of natural and/or synthesized glycans in one experimental setting. They are highly sensitive and significantly reduce reagent consumption.[36,37]

3.1 Glycan binding proteins

The generation of glycan microarrays with high specificities toward glycan-binding proteins is important both for investigating their biological functions and for analytical applications in biomedical and diagnostic fields. Lectins specifically recognize carbohydrate motifs[38] and Table 1 shows the primary sugar specificities of some important lectins. Please note that most lectins have additional structural requirements for binding.

Taylor and Drickamer[39] reported that mannose binding protein (a C-type lectin) did not bind to a glycan array even though the array contained putative ligands and the protein was active in other assays. Based on that, they suggested that the array did not achieve sufficient density to support multivalent

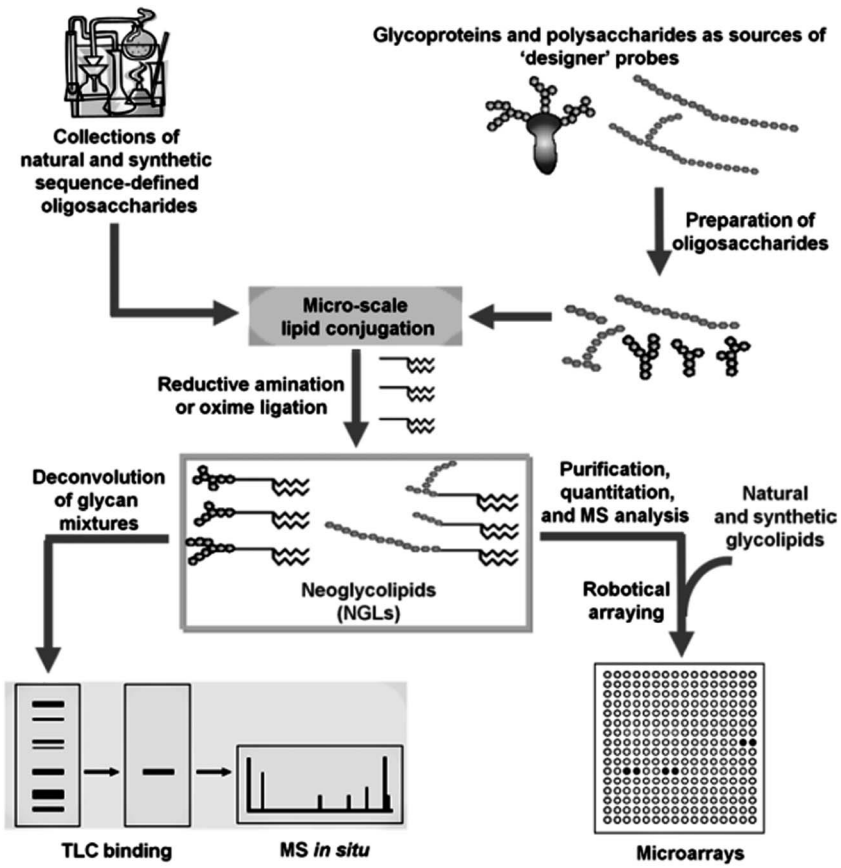

Fig. 2 Fabrication diagram of a neoglycolipid based oligosaccharide array platform. Reproduced with permission from Ref. 32.

Table 1 Lectin specificities.

Sugar	Lectin[a]
Fucose	AAL, LTL, UEA I
Galactose	ACL, ECL, EEL, GSL I, GSL I–B_4, Jacalin, MAL I, PNA, RCA I, RCA II, SBA
Glucose	Con A, LCA, PSA
Mannose	Con A, GNL, HHL, LCA, NPA, PSA
N-Acetylgalactosamine	BPL, DBA, GSL I, MPL, PTL, RCA I, RCA II, SJA, SBA, VVA, WFA
N-Acetylglucosamine	DSL, GSL II, LEL, STL, WGA
Sialic acid	MAL II, SNA
Complex structures	PHA-E, PHA-L

[a] AAL: Aleuria Aurantia Lectin; LTL: Lotus Tetragonolobus Lectin; UEA I: Ulex Europaeus Agglutinin I; ACL: Amaranthus Caudatus Lectin; ECL: Erythrina Cristagalli Lectin; EEL: ~~Erythrina Cristagalli Lectin~~; GSL I: Griffonia (Bandeiraea) Simplicifolia Lectin I; MAL I: Maackia Amurensis Lectin I; PNA: Peanut Agglutinin; RCA I: Ricinus Communis Agglutinin I; RCA II: Ricinus Communis Agglutinin II; SBA: Soybean Agglutinin; ConA: Concanavalin A; LCA: Lens Culinaris Agglutinin; PSA: Pisum Sativum Agglutinin; GNL: Galanthus Nivalis Lectin; HHL: Hippeastrum Hybrid Lectin; NPL: Narcissus Pseudonarcissus Lectin; BPL: Bauhinia Purpurea Lectin; DBA: Dolichos Biflorus Agglutinin; MPL: Maclura Pomifera Lectin; PTL: Psophocarpus Tetragonolobus Lectin; SJA: Sophora Japonica Agglutinin; VVA: Vicia Villosa Lectin; WFA: Wisteria Floribunda Lectin; DSL: Datura Stramonium Lectin; GSL II: Griffonia (Bandeiraea) Simplicifolia Lectin II; LEL: Lycopersicon Esculentum (Tomato) Lectin; STL: Solanum Tuberosum (Potato) Lectin; WGA: Wheat Germ Agglutinin; MAL II: Maackia Amurensis Lectin II; SNA: Sambucus Nigra Lectin; PHA-E: Phaseolus vulgaris Erythroagglutinin; PHA-L: Phaseolus vulgaris Leucoagglutinin. Reproduced with permission from http://www.vectorlabs.com/catalog.aspx?catID=31

binding. In another instance, binding of a plant lectin, conconavalin A, showed equivalent binding to high and low affinity mannose containing ligands when printed in high density, but bound only to the high affinity glycan printed at reduced density.[40] Such examples document the need for more systematic investigations into glycan presentation in different array formats.

Proteoglycans, ~~glycoshingolipids~~, and glycoproteins are the major classes of mammalian glycans. There are numerous mammalian glycan binding proteins (GBPs) outside of these three major families, and new GBPs are continuously being discovered. Notable results from the analysis of glycan arrays include: M-ficolin, a soluble serum protein which is involved in innate immunity was shown to bind sialylated glycans;[40] and malectin, an ER protein whose function was unknown until the demonstration of its binding to a $Glc_3Man_9GlcNAc_2$-N-linked glycan, suggesting that it is involved in the processing of *N*-linked glycan intermediates in the biosynthetic pathway.[41]

Numerous plant lectins have been identified and have been demonstrated to have highly diverse specificities for glycan ligands. They are widely recognized as being important tools in glycobiology research,[42,43] and lectins with novel specificities continue to be identified and characterized. Glycan microarrays have now become principle tools for defining the detailed specificities of plant lectins.[44]

3.2 The mannose receptor

The mannose receptor (MR) is one of the best described members of the type I transmembrane C-type lectins. MR has been considered to be a potential entry receptor for a variety of pathogens, and a target for regulation by human pathogen-associated proteins suggesting relevance for this receptor in the context of some human diseases. It also assists in the clearance of pathogens, the capture of foreign antigens for presentation to MHC-II compartments, the clearance of glycoprotein hormones and extracellular peroxidases, endocytosis of lysosomal acid phosphatase, as well as in the regulation of glycoprotein homeostasis.[45,46]

Glycan microarray applications have been successfully used in the analysis of cells for mannose receptor (MR) expression. One of the surface receptors of MR recognizes extracellular host and foreign substances with exposed terminal mannose residues. Through this binding, the MR mediates the internalization of a wide range of pathogens and host-derived molecules, influences inflammation; and mediates clearance of apoptotic cells, endogenous hydrolases, and peroxidases.[46] Understanding the molecular and cell biology of this receptor could be key to develop strategies to contain infection and control inflammation.

3.3 Infectious diseases

Glycoarrays are being used more and more to extensively study infectious diseases. Viruses and bacteria utilize the surface carbohydrates of human cells as initial recognition and attachment sites.[47,48] The hemagglutinins on the surface of influenza viruses mediate attachment to the host cell by binding sialylated carbohydrates and different influenza variants vary in their sialic acid binding profile.[49] A good example of the use of glycan microarrays to identify influenza virus subtypes from infected serum samples is to analyze their sugar binding specificities.[50] Fast glycoarray tests might detect influenza strains in the early stages of an epidemic infection and identify changes in the carbohydrate binding profiles of dangerous virus mutations.[41] Another use of glycoarray tests is a panel of *Salmonella*-related carbohydrate antigens employed to analyze human sera from salmonellosis and healthy patients.[51]

Glycosylphosphatidylinositol (GPI) glycolipids are present in the plasma membrane of *Plasmodium falciparum*, a parasite that causes malaria in humans. GPI is an important toxin and humans in malaria endemic regions often produce high levels of anti-GPI antibodies. Synthetic GPI glycoarrays have been

used to establish the binding specificity measurements for anti-GPI antibodies, correlate antibody levels and protection from severe malaria and aid efficient carbohydrate-based antitoxin vaccine design.[47]

3.4 Analysis of phosphorylated sugar structures

A special and important subset of glycan microarrays has been developed for the analysis of phosphorylated sugar structures. The specificity of cation-dependent and -independent mannose 6-phosphate receptors (CD-MPR and CI-MPR) for high mannose-type *N*-glycans of defined structures containing zero, one, or two Man-P-GlcNAc phosphodiester or Man-6-P phosphomonoester residues was determined by a phosphorylated glycan microarray.[52] The difficulty in defining the specific interactions of P-type lectins with glycans containing Man-6-P or Man-P-GlcNAc was due to the lack of defined glycan structures for direct binding or inhibition studies.[34]

Song *et al.*[26] have exploited a bifunctional fluorescent linker, 2-amino-*N*-(2-aminoethyl)-benzamide (AEAB), to generate a library of fluorescently labeled high mannose-type *N*-glycans, prepared from bovine ribonuclease B and soybean agglutinin (Fig. 3). The glycans were purified, characterized, and enzymatically modified by a recombinant GlcNAc-phosphotransferase to contain Man-P-GlcNAc phosphodiesters. The amine-activated glycans were printed and covalently bound on *N*-hydroxysuccinimide-activated glass slides followed by ~~treatment~~ with different concentrations of CD-MPR or CI-MPR.[26,30] Using this novel fluorescent derivatization method, many commercially available free reducing glycans could be derivatized.

3.5 Clinical applications

The clinical application of glycan microarrays has risen sharply in recent years. A good example of this is the use of Keyhole limpet hemocyanin (KLH), a large molecular weight glycoprotein of marine origin, which is extensively utilized for basic research and clinical applications of glycan microarray techniques. Due to its high immunogenicity and low toxicity, KLH has become the standard carrier protein for making monoclonal and polyclonal antibodies against such small molecule haptens as oligosaccharides.[53] Several peptide-KLH conjugates and carbohydrate-KLH conjugates have even progressed to clinical trials as cancer vaccines.[54,55]

A carbohydrate microarray method has also been used to profile the antibody responses in 14 individuals immunized with KLH plus alum adjuvant.[56] However, while the appropriate immune response to carbohydrate antigens is a major component of this method, it may vary significantly from person to person. Apparently, glycan microarrays have been useful for evaluating such responses.[57] Another interesting development in the field has been the high-throughput screening of inhibitors to block carbohydrate-mediated molecular recognition in specific biomedical applications, also utilizing carbohydrate microarray methods.[58]

In the work of Lawrie *et al.*,[59] a glycan microarray containing 37 covalently bound carbohydrates was used to identify sugar structures that triggered a humoral immune response in classical Hodgkin's lymphoma (cHL) patients. This carbohydrate microarray enabled the investigation of glycan variations of specific proteins in patient populations, also in response to changing conditions.[5]

4 Conclusions

In recent years, glycoarrays have become standard tools for screening a great number of sugar–biomolecule interactions and for investigating the role of carbohydrates in biological systems, especially in large scale diagnostic applications. Glycan microarrays have been applied in the determination of

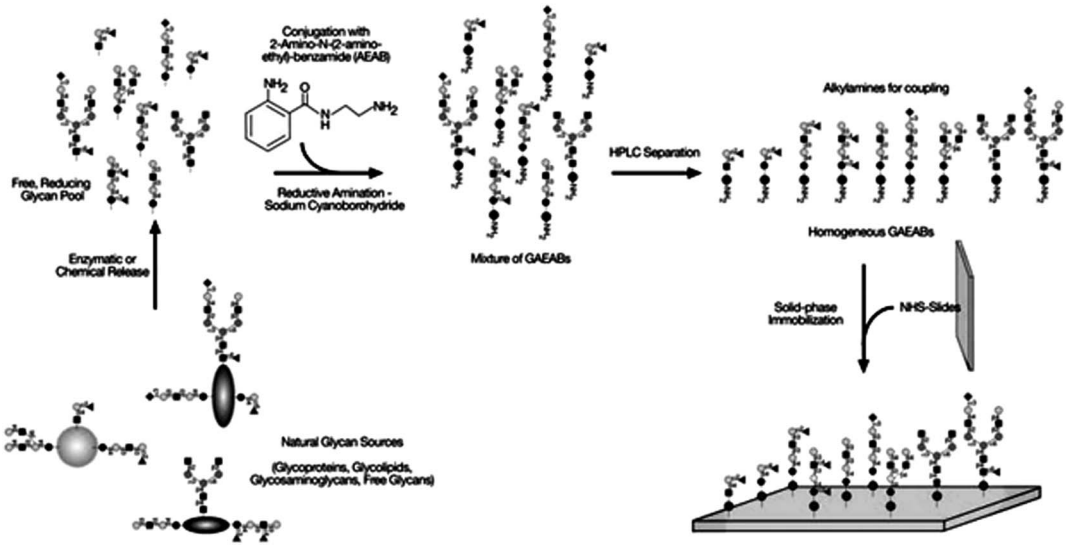

Fig. 3 The general strategy of natural glycan array development using complex glycoconjugates as starting materials. Reproduced with permission from Ref. 30.

the sugar-binding profiles of proteins, nucleic acids, whole cells, and pathogens, just to mention the most important ones, and have provided valuable information for the design of carbohydrate-based drugs. Glycan arrays also constitute an attractive platform for the analysis of serum samples, opening up the way for a wide variety of biomedical applications, such as the identification of novel sugar based biomarkers to diagnose early-stage diseases and the detection and diagnosis of pathogenic infections.[47]

Natural glycans can be utilized as an almost unlimited source of glycan structures.[51] The recognition of complex carbohydrates by glycan binding proteins is a good way of interpreting the glycome and providing information on its involvement in biological processes. However, modern glycomic research shows that each cell and tissue of every organism generates a wide variety of glycans and each cell type has a distinctive repertoire of glycan structures. In addition, hundreds of glycan binding proteins have been identified and many more are predicted to be discovered.[30]

Currently, microarrays are primarily used as research tools, however, they will quickly find their place in large scale biomedical applications. Sugar microarrays represent a promising platform to shed light on structure–function relationships of sugar–sugar, sugar–protein, sugar–lipid, *etc.*, interactions at the global level. The incubation of glycoarrays with serum or plasma samples opens up new avenues for novel medically relevant applications, including the identification of carbohydrate cancer markers and specific identifications of pathogen infections. The identification of markers in early-stage cancers could lead to improved therapies and survival rates of patients.

In recent years a number of glycan microarray platforms, based on the specific (covalent or non-covalent) immobilization of chemically conjugated glycans on a modified surface, have been developed for high-throughput glycomic research. Carbohydrate arrays vary in ligand presentation, glycan origin (chemically synthesized or isolated from natural sources), assay conditions, detection method, microsphere based suspension array (SA) and immobilization on flat surfaces (printed glycan array, ELISA); all of which contribute to the affinity and selectivity of the binding.

With the aim of automating the synthesis of complex carbohydrates, a number of sophisticated chemical methodologies have been developed for the rapid generation of glycan libraries. The approach has been pioneered by Seeberger's group[60] utilizing solid-phase synthesis to carry out the iterative glycosylation and deprotection steps. They have capitalized on this method, along with more traditional solution-phase synthesis, to create libraries of heparin sulfate glycans and high-mannose oligosaccharides.[8,61,62] Another elegant technology is the Optimer-based one-pot, solution-phase oligosaccharide synthesis method.[63] In this system, a computer program is used to select the appropriate glycosyl donor and acceptor building blocks such that when added sequentially to a reaction vessel the desired oligosaccharide is assembled.

Future perspectives

Miniaturized methodologies in microarray formats are particularly promising in the fields of biomedical research and clinical diagnostics. While DNA microarray technology has the advantage of utilizing the polymerase chain reaction (PCR) based amplification and cloning strategies to produce high quantities of nucleotides even if starting from just a few copies, protein and carbohydrate microarrays currently lack such amplification techniques facing a limited sample availability issue. While protein microarrays usually utilize antibodies for interrogation, carbohydrate microarrays can use either antibodies or special carbohydrate-binding proteins (*e.g.*, lectins) to detect binding. Both approaches can be used in diagnostic and/or prognostic applications or to characterize carbohydrate-cell or carbohydrate-microbe recognition events.[64]

There are myriads of possibilities for the use of microarray technologies in biomedical and diagnostic applications.[21] During the last decade, glycan microarrays have moved from a proof of principle concept to a powerful glycobiology research toolset. Despite the documented utilization of glycan microarrays, the progress to date represents only the beginning. One of the challenges in the interpretation of glycan array data is to determine the specific features of glycan structures that are critical for binding. Microarrays have been useful tools for determining the specificity of glycan binding proteins and certain surface oligosaccharides that might be an important element for cell–cell communication.

The identification of all the glycan structures associated with particular cells is underway. Databases containing published glycan sequences are already providing information on the scale and diversity of the glycome. It has been estimated that 500–600 endogenous unique glycan structures are present in glycoproteins and glycolipids in mammalian systems. Expansion of the glycan content of glycan microarrays to cover the majority of known glycans will therefore be one of the most important challenges for the next few years. Bacterial polysaccharides and other pathogen and microorganism related glycans will certainly be involved in future array designs as well.[12]

Abbreviations

AEAB	2-Amino-*N*-(2-aminoethyl)-benzamide
CD-MPR	Cation-dependent mannose 6-phosphate receptor
CI-MPR	Cation-independent mannose 6-phosphate receptor
ER	Endoplasmic reticulum
GBP	Glycan binding protein
GPI	Glycosylphosphatidylinositol
MPR	Mannose 6-phosphate receptor
MR	Mannose receptor
PGA	Printed glycan array
SA	Suspension array
TGL	Tagged glycan libraries

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