New development of glycan arrays

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Received 5th February 2009
First published as an Advance Article on the web 27th April 2009
DOI: 10.1039/b902510n

The development of glycan arrays has enabled the high-sensitivity and high-throughput analysis of carbohydrate–protein interactions and contributed to significant advances in glycomics. A number of new array platforms that allow for qualitative and quantitative analysis of mono- and multivalent interactions on surfaces have been developed recently. Glycan arrays are not only a powerful tool for basic research, but also a promising technique for medical diagnosis, and detection of pathogens and cancers. These studies also have led to the design of efficient carbohydrate-based antimicrobial or anticancer vaccines.

Introduction

Among the three major classes of macro-biomolecules—nucleic acids, proteins, and carbohydrates—the carbohydrates are the least studied and understood. For a long time, biochemical research on carbohydrates has been focused on dietary sugars and their metabolic pathways. In contrast, the oligo- and polysaccharide functions in organisms are less known despite their importance and ubiquitous presence. Based on their sequences, more than 50% of human proteins are predicted to be glycosylated. Carbohydrates often exist on cell surfaces as glycoprotein or glycolipid conjugates and play important structural and functional roles in numerous biological recognition processes, for example, protein folding and stabilization, viral and bacterial infection, cancer metastasis, inflammatory response, innate and adaptive immunity, and many other receptor mediated signaling processes.[1–3] Moreover, there exist many examples in which glycosylation is required for biological activity. Furthermore, many organisms such as sessile plants have evolved specific glycosylation mechanisms to detoxify harmful exogenous xenobiotics.[4] Despite the increasing awareness of the biological significance of carbohydrates, the study of carbohydrate–protein interactions still encounters much difficulty. This is largely because of the structural complexity and synthetic difficulty of carbohydrates and the low affinity of their interactions with glycan-binding proteins (GBPs). Typically the monomeric dissociation constant (K_D) in a carbohydrate–protein interaction is in the millimolar range; thus, carbohydrate-mediated biological responses often occur through multivalent interactions on the cell surface in order to achieve high affinity and specificity.[5] A major challenge in cell biology is to define the interactions of oligosaccharides and proteins involved in many biological processes. However, pure oligosaccharides are difficult to obtain and there is a need for the development of highly sensitive and high-throughput methods for identification and binding study of carbohydrates recognized by various receptors. In the past year, glycan arrays have been developed and become

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a powerful platform to serve this purpose.[6–15] In general, the chip-based format bears many advantages: 1. Only small amounts of sugar are required for arraying, overcoming the shortage of pure carbohydrates; 2. Carbohydrates on the plate are multivalent and therefore mimic their natural presentation on the cell surface.

Glycan array fabrication and detection

Among different immobilization methods, covalent attachment and physical adsorption are two major strategies. Fluorescence-based detection is the predominant method for analysis of protein–carbohydrate interactions. Till now, many excellent reviews have emphasized the methods of fabrication.[16–19] Here we will focus not only on the new fabrication methods developed after 2005, but also on new detection methods.

Immobilization of glycans on properly derivatized surfaces is the most employed method of fabricating glycan microarrays. However, the difficulty of obtaining various modified glycans has slowed down the development of glycan microarrays. To avoid multiple synthetic reactions, one-step methods for the modification of free sugars with proper linkers have been exploited. For example, simple carbohydrates or oligosaccharides, when reacted with *N*-methylaminooxy-containing bifunctional linker produced a cyclic adduct in the β-configuration.[20–21] Furthermore, in order to avoid the requirement for modification of glycans, the hydrazine- and aminooxy- derivatized surfaces have been developed to immobilize the unmodified sugars.[22–24] Acyclic modifications are formed when an aminooxy surface is used. In contrast, unmodified glycans bind to hydrazide modified surfaces to form cyclic structures with β-configurations at their anomeric positions, a type of array more similar to the natural form. Prof. Sprenger *et al.* used photolabile aryltrifluoromethyldiazirine group coated surface to covalently attach unmodified sugar by UV irradiation.[25] A major drawback of this technique is the nonspecific attachment of glycans to the surface owing to the nonselective nature of the carbene reactions. Click chemistry has been used to create covalent glycan arrays on the microtiter plate.[26] Recently, Prof. Ravoo and Michel used the microcontact printing technology that is an emerging fabrication method for protein and DNA arrays to create glycan arrays on glass and Si wafers *via* a click reaction. The advantages of microcontact printing include high edge resolution (better than 100 nm), reproducible probe density, and homogeneous spots.[27]

Glycan arrays are a powerful tool for studying glycobiology and the high-throughput bioassay of epidemic diseases.[28–31] However, it is difficult to characterize and quantify the immobilized oligosaccharides on the array surface. To tackle this problem, Prof. Cummings *et al.* has developed a smart method by using 2,6-diaminopyridine (DAP) as a fluorescent linker to first conjugate unmodified sugar and then print the glycan–DAP conjugates (GDAPs) on epoxy-activated slides, which permits their quantification and positioning directly on printed glycan microarrays using a commonly available fluorescent slide scanner. However, this method requires reductive amination that can result in a loss of structural information at the reducing end of the free glycan.[32–34] Combining array and mass spectrometry technologies has opened a new avenue for this field. Wong *et al.* have recently demonstrated that, by using the desorption/ionization on silicon mass spectroscopy (DIOS-MS) technique, the oligosaccharides that are immobilized *via* covalent bonding on porous silicon with a photo-cleavable linker can be identified and characterized.[35] Preparation of porous silicon plates, however, requires the usage of corrosive acid, and the conventional glass slides cannot be used for mass spectrometry study. Thus, easily prepared and environmentally friendly aluminium coated glass (ACG) slides have been developed recently.[36] Glycans arrayed with a photo-cleavable linker on the ACG slide surface can be subjected to both time-of-flight mass spectrometry (MS-TOF) analysis, and fluorescence-tagged protein binding evaluation with higher sensitivity. However, this approach results in a relatively low signal-to-noise (S/N) ratio in mass detection, most likely due to incomplete photo-cleavage. In addition, the method required more synthetic steps to install the photo-cleavable linker to the glycan array. Recently, Pohl *et al.* developed a new type of glycan array based on noncovalent fluorous-phase immobilization.[37] Siuzdak's group also reported the use of nanostructure-initiator mass spectrometry (NIMS),[38] a unique approach to laser desorption/ionization based on liquid ('initiator')-coated nano-structured surfaces for spatially defined mass analysis. Combined with these technologies, a NIMS enzymatic (Nimzyme) assay[39] was developed to immobilize the substrate on the surface by using fluorous-phase interactions. The "soft" immobilization allows efficient desorption/ionization while enabling the use of surface-washing steps, for the preferential retention of the tagged products and reactants. The Nimzyme assay is sensitive to sub-picogram levels of enzyme, can detect both addition and cleavage reactions, is applicable over a wide range of pHs and temperatures, and can measure activity directly from crude cell lysates.[39] Thereafter, Wong's laboratory has further modified the ACG slide to a teflon-like ACG surface, and created a new generation of glycan array on this surface (Fig. 1). This new array did not require the use of matrix and was demonstrated to be a powerful tool to study cellulase activities and specificities.[40] Laurent *et al.* used MALDI-TOF mass spectrometry to monitor the enzymatic synthesis of mucin-type glycopeptide arrays on gold surfaces using a polypeptide GalNAc-transferase.[41] They also used the same strategy to assemble a glycan array to assess the activity

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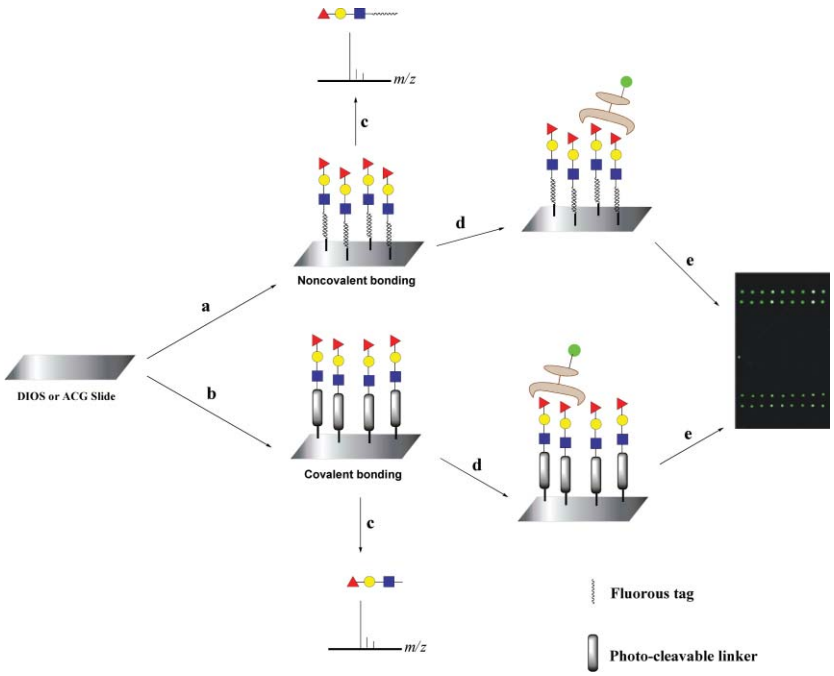

Fig. 1 New generation of glycan arrays on DIOS or ACG slides: they allow the oligosaccharides immobilized on the supporting substrate surface to be characterized and quantified using mass spectrometry. (a) Development of noncovalent bonding array by using fluorous-phase interaction. (b) Covalent array with a photo-cleavable linker. (c) Characterization and quantification of the oligosaccharides immobilized on the supporting surface by using mass spectrometry. (d) Detection by fluorescence-tagged protein. (e) Readout by fluorescence scanner.

and specificity of bovine α1,4-GalT activity and specificity in a label-free manner by using MALDI-TOF mass spectrometry.[42]

Beside mass spectrometry and fluorescence microscopy, surface plasmon resonance (SPR) has also been used as a detection method.[7,43–48] This method does not require specially labelled reagents,[7] and the relatively weak multivalent carbohydrate interactions can be detected.[49]

Quantitative detection on the surface

Most binding experiments with glycan arrays have been performed at one or two concentrations of proteins to globally profile carbohydrate–protein interactions. Although qualitative assessment of binding by this method is useful, using a one-point threshold-based approach to assess binding affinities cannot accurately reflect the relative binding strength. Values are highly variable from batch to batch because the spot intensities depend on immobilization efficiency. Recently, the Wong lab,[50] and the Shin lab[23] have developed a quantitative method to assess the binding affinity between carbohydrates and proteins. A series of protein concentrations were incubated with repeated subarrays which contained several carbohydrates to get surface dissociations ($K_{D,surf}$) from a single experiment. The $K_{D,surf}$ values obtained from these experiments were found to be in a good agreement with those detected by SPR.[51] By varying the printing concentration of glycans, multivalent interactions were probed. Higher density printing generally led to lower observed $K_{D,surf}$, indicating a multivalency effect which mimics the multivalent display of glycans on cell surfaces (Fig. 2).[50] Later, this approach was applied to determine the $K_{D,surf}$ values of Globo H and its truncated derivatives with Globo H monoclonal antibodies MBr-1 and

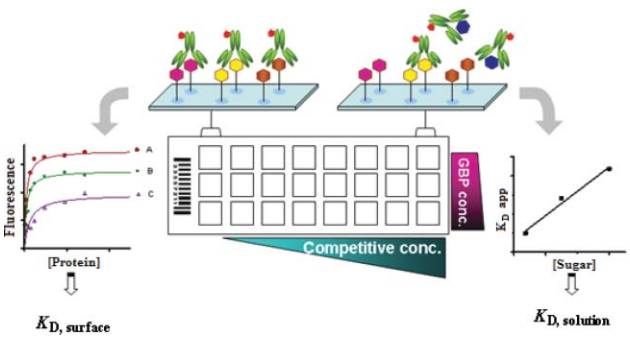

Fig. 2 Quantitative analysis of protein–carbohydrate interactions to obtain surface and solution dissociation constants from glycan arrays.

VK-9,[52] as well as polymannose with human HIV monoclonal antibody 2G12.[53] This method was also used to confirm the efficiency of fabrication of glycan arrays. For example, Pohl *et al.* studied the interactions of mannose-related structures with Con A,[21,54] and demonstrated that the noncovalent fluorous-tagged surface was useful for quantitative assessment of carbohydrate–protein interactions. In the case of neoglycoconjugates generated from carbohydrates and protein *via* reductive amination,[55] it was shown that the carbohydrate bioactivities of neoglycoconjugates were still retained. Moreover, the solution dissociation constants (K_D)[50] and IC_{50} values[7,23] of inhibitors or carbohydrates were determined by competition using the inhibitors or carbohydrates in solution. Using this method, the K_D values of a series of α-GalCer derivatives interacting with CD1d were measured.[56] The study revealed that the cell-based cytokine secretion profile was well correlated with the stability of the complex of CD1d-glycolipids. It is apparent that the quantitative analysis of

carbohydrate and protein interaction by glycan array is useful for characterization of the sugar-binding specificities of proteins and for the high-throughput discovery of inhibitors of carbohydrate-binding proteins of therapeutic interest.

Application for infectious disease studies

Most pathogens contain specific polysaccharides on their cell surface, which can elicit antibody responses in infected humans. Microbial polysaccharide microarrays can be used for detection of pathogen infection by analyzing patient serum samples. Wang and Lu used glycan arrays to characterize the carbohydrate-binding activity of SARS-CoV neutralizing antibodies induced by an inactivated SARS-CoV vaccine and found a cross reactivity between the inactivated SARS-CoV vaccine and a host carbohydrate.[57] They also discovered that rabbit IgG antibodies elicited by *Bacillus anthracis* spores specifically recognize a rhamnose tetrasaccharide chain that decorates the outermost surface of the *B. anthracis* exosporium.[58] This tetrasaccharide appears to be a key biomarker of *B. anthracis* spores. In an effort directed toward the development of an AIDS vaccine, glycan arrays were used to dissect the glycan-binding specificity of the HIV-1 broadly neutralizing antibody 2G12.[59,60] Employing the same strategy, a polysaccharide microarray to detect bacterial infection using human or animal serum samples was prepared by immobilizing bacterial polysaccharides.[61,62] Parthasarathy *et al.* used bacterial 'signature' carbohydrate arrays to detect and differentiate *B. pseudomallei*, *B. anthracis* and *F. tularensis* antibodies in infected patients, and infected or vaccinated animals.[63] More recently, Seeberger's group created a synthetic parasite glycosylphosphatidylinositol (GPI) array[64] to study the immune response of people living in malaria-endemic regions. They showed that only the largest GPI structures bound anti-GPI antibodies and the binding specificities of anti-GPI antibodies varied between individuals. An array of all phosphatidylinositol mannose (PIM) glycans from *Mycobacterium tuberculosis* was also developed by Seeberger's group to investigate the function of PIMs as potential antigens.[65] Blixt *et al.* reported an array containing oligosaccharide antigens specifically expressed by serogroups *Salmonella enterica* sv. Paratyphi, Typhimurium, and Enteritidis.[66] This glycan array was used to detect the antibody from patients with salmonellosis. These studies have led to the design of carbohydrate-based vaccines (Fig. 3).

Carbohydrates on the surface of human cells are the initial recognition and attachment sites for viruses and bacteria.[67] The surface glycoprotein hemagglutinin (HA) on influenza A viruses binds to glycans with terminal sialic acids. There are reports on the specificity of hemagglutinin (HA) from avian and human influenza sources, including those reconstructed from past pandemic strains.[68–70] Binding analysis of HA variants recovered from pandemic and circulating strains on a 260-member glycan array demonstrated differences in the recognition of carbohydrate linkages. In general, human influenza viruses preferentially bind to the Neu5Acα2,6Gal residues and avian influenza viruses are specific for Neu5Acα2,3Gal residues. In addition, sulfation and fucosylation will affect the binding affinity. Remarkably, 1918 pandemic HA switched specificity to human epithelial cells, a change from α-2,3 to α-2,6 NeuAcα-Gal-binding preference with only two amino acid substitutions.[71,72] These findings provide

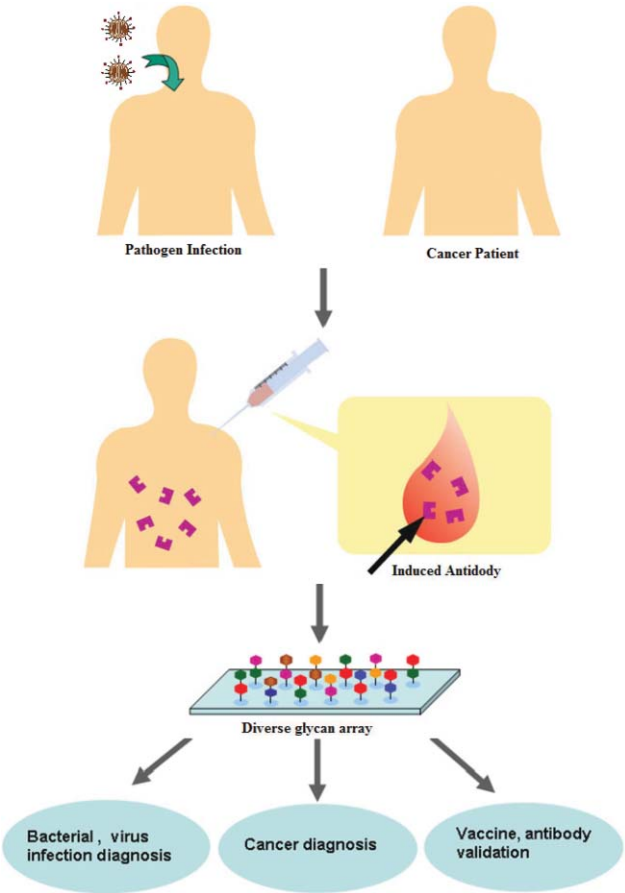

Fig. 3 Glycan-binding specificity profiling for the diagnosis and detection of disease state or antibody.

information with regard to the host–virus interaction associated with different influenza strains and their evolution.

An array of monosaccharides was also used for binding study of *Escherichia coli* ORN178. It was found that *E. coli* specifically bind to mannose-containing slides.[73] By using glycoconjugate arrays, the Ruhl group has characterized the adhesion specificities of *Helicobacter pylori* and other bacteria.[74] These studies point to the possibility of using carbohydrate microarrays as a detection system for pathogens.

Application for cancer studies

Aberrant glycosylation is one of the hallmarks of cancer progression. Cancer-associated carbohydrate antigens are often found on the surface of cancer cells. Understanding their roles in cancer progression will lead to the development of new therapeutics and high-sensitivity diagnostics for cancers. Globo H is a hexasaccharide which is a member of a family of antigenic carbohydrates that are highly expressed on various types of cancers, especially cancers of the breast, prostate, pancreas, colon and lung.[75] The Globo H hexasaccharide cancer marker and nine structural analogs were arrayed by Wong's group and used to analyze monoclonal antibodies Mbr-1 (a mouse IgM anti-Globo H monoclonal antibody), VK-9 (a mouse IgG anti-Globo H monoclonal antibody),[76] plasma samples of 58 breast cancer patients and 47 healthy blood donors. The authors have found that both the levels of IgG and IgM

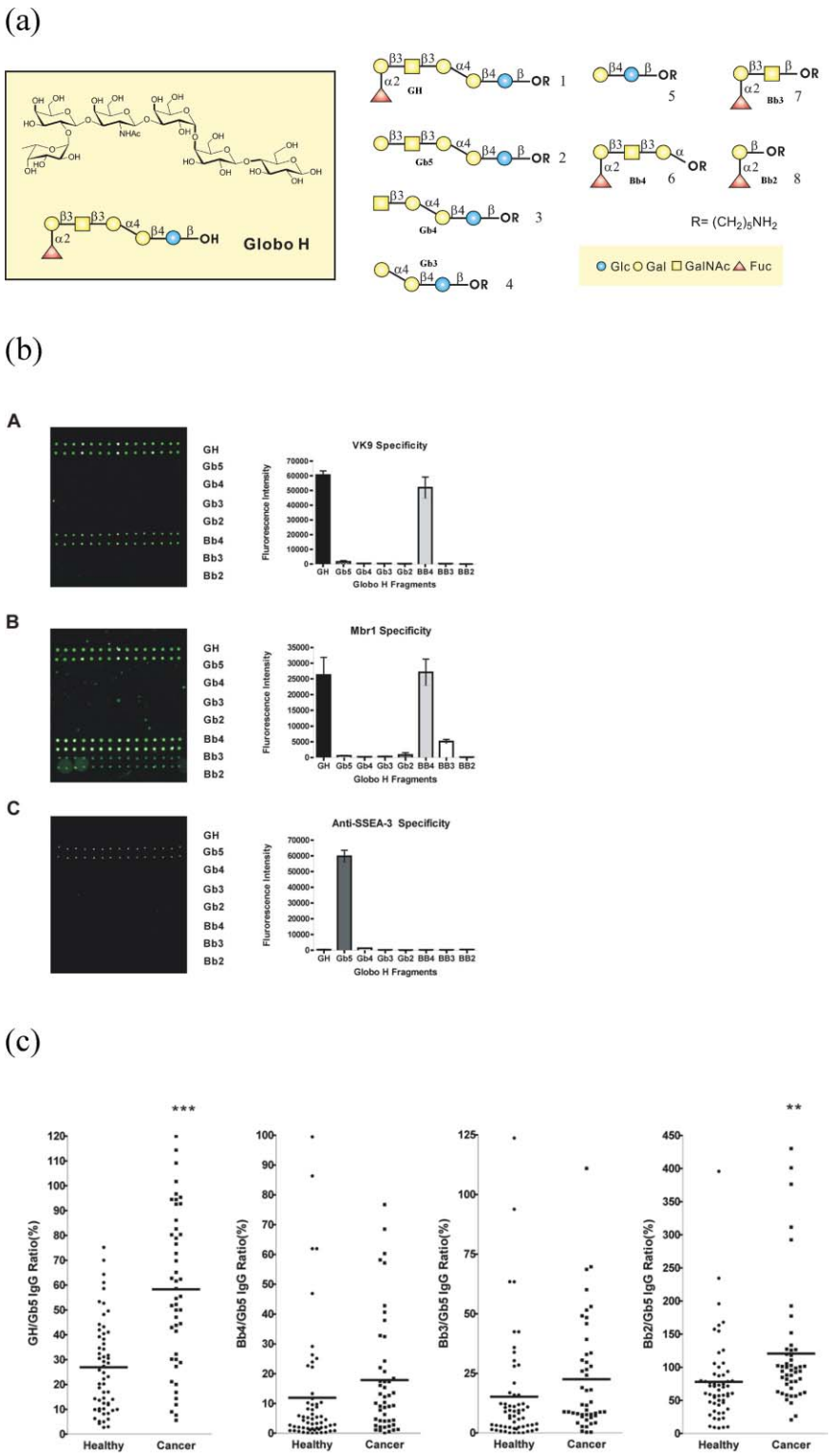

Fig. 4 Array of Globo H and analogs for breast cancer study. (a) Chemical structure of Globo H and abbreviations of Globo H analogs. (b) Binding of monoclonal antibodies to Globo H and its analogs [(A) Slide image obtained from fluorescence scan after antibody incubation assay with VK-9. The grid contains sugars 1–8 printed at 80 μM concentration. Slide images obtained by assay with MBr1 (B) and anti-SSEA-3 monoclonal antibody (C)]. (c) Ratios of IgG levels against Globo H analogs in sera from breast cancer patients and normal blood donors. The relative fluorescence ratios were obtained from the fluorescence intensity of Globo H or Globo H analogs divided by the fluorescence intensity of Gb5. The mean of the Globo H/Gb5 IgG ratios was significantly higher in the sera of breast cancer patients.

against Globo H were significantly higher in breast cancer patients than in normal individuals (P <0.0001) (Fig. 4).[52] They have also compared the array method with the traditional ELISA method and found that the array method required only attomol amounts of materials and is more effective and 5 orders of magnitude more sensitive. However, this result is in contrast to Bovin *et al.*'s study[77] using biotinylated glycoconjugates attached to streptavidin coated on gold as the glycan microarray and 96-well plates coated with sugar-polyacrylamide (Sug-PAA) for the ELISA assay. Surprisingly, they found that the overall sensitivity of the glycan microarray was lower than the ELISA method in the comparative assay. This difference may be caused by a lower density array. Lawrie *et al.* used a commercially available array of 37 different carbohydrates to profile Hodgkin's lymphoma sera and showed a marked deviation in glycan-binding specificity compared to normal samples.[78] Another strategy that used lectin-affinity purification and natural glycoprotein microarrays to screen different glycosylation patterns between healthy and different stages of pancreatic cancer was developed by Lubman's group.[79] Combined with the lectin array developed by Prof. Mahal *et al.* firstly,[80] glycan array profiling is expected to facilitate the identification of specific biomarkers, which can be added to the currently used DNA and protein biomarkers for development of diagnostics.

Application of glycosaminoglycan array

Glycosaminoglycans (GAGs) are large and complex classes of carbohydrates, including chondroitin sulfate, heparin/heparin sulfate, dermatan sulfate, keratin sulfate, and hyaluronan. GAGs are known to play a key role in regulating growth factors, virus entry, and angiogenesis;[81] however, their structure–activity relationships are poorly understood. Seeberger *et al.* designed the first microarray of heparin-like glycosaminoglycans to tackle this problem.[82] The array was incubated with fibroblast growth factors (EGF-1 and EGF-2), and it was found that EGF-1 not only interacted with the hexamer and tetramer of heparin oligosaccharides but also with the unusual 2,4-*O*-sulfated monomer. The same group also used the microarray containing a small library of synthetic heparin oligosaccharides to profile eight chemokines (CCL21, IL-8, CXCL12, CXCL13, CCL19, CCL25, CCL28, and CXCL16),[83] and SPR experiments were performed to validate the carbohydrate microarray binding results. The Hsieh-Wilson group reported the use of a chondroitin sulfate microarray to probe the specificity of TNF-α,[84] as well as midkine-derived and brain-derived neurotrophic factors.[85] The tetrasulfated tetrasaccharide CS-E was found to react strongly with these growth factors within the physiological concentration range. A brain neuron growth experiment confirmed that the CS-E motif stimulated neurite outgrowth by about 50%.[85] More recently,[86] Buzás *et al.* created a GAG array, by using the carbohydrate components of proteoglycans released from degrading cartilage, to detect anti-GAG antibodies of the sera from rheumatoid arthritis patients (n = 66), umbilical cord serum samples (n = 11) and adult controls (n = 54). They found that anti-GAG antibodies were absent in the umbilical cord sera. On the other hand, they were readily detectable in adult controls and were significantly elevated in patients with rheumatoid arthritis (p <0.001). The highly abundant and cross-reactive GAG-specific natural auto-antibodies in serum may serve as novel disease-state markers in patients with rheumatoid arthritis.[86] It is anticipated that these microarrays will accelerate the understanding of GAG-proteins and pinpoint the sulfation patterns responsible for modulating physiological and disease states. These microarrays also provide valuable structure information for the design of inhibitors or antagonists of these therapeutically important cytokines, growth factors and rheumatoid arthritis.

Consortium for Functional Glycomics (CFG)

The CFG is a large research initiative to understand the role of carbohydrate protein interactions at the cell surface in cell–cell communication (www.functionalglycomics.org).[87] In the landmark studies by researchers at the Consortium for Functional Glycomics, a comprehensive array of more than 300 glycans on a glass slide was used to analyze the specific binding of mammalian, plant, viral, and bacterial lectins.[15]

Summary

Glycan arrays are being developed to decode the information content of the glycome. They are an important tool for studying carbohydrate–protein interactions and glycoenzyme specificities in a high-throughput fashion, both qualitatively and quantitatively. In the future, glycoarrays may be used to profile the glycosylation pattern of tumor cells and their non-cancerous counterparts to provide information regarding signaling events, regulation, cellular transport, catalytic activity, targeting, protein fusion and binding, and other biological reactions. In addition, glycan arrays can be used to detect the presence of antibodies, T lymphocytes, or other immune cells that recognize antigens associated with cancer and pathogens. One such antigen that is commonly over-expressed in breast cancer is Globo H, as previously mentioned. Conjugating Globo H, or sections of the hexasaccharide, to carrier proteins induce a humoral response in humans to the saccharide, indicating its potential utility as cancer vaccine. Glycan arrays can be used to monitor the level of antibody in the blood after vaccination. The advances in the chemical and enzymatic synthesis of complex oligosaccharides will greatly facilitate the rapid development of glycan arrays for use in biomedical study.

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