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1 Introduction

Carbohydrates and their glycoconjugates are known to play important roles in a variety of biological processes and a wide range of diseases. They are involved in numerous biological recognition events, inflammation, cancer development and metastasis, and bacterial and viral infections.\textsuperscript{1–4} Studying the biological significances of carbohydrates, in particularly their roles in disease progression, are proven very challenging due in part to their structural diversity, the limited access to complex carbohydrate-containing molecules, and the lack of proper tools that enable the high-throughput analysis of carbohydrates interactions with other biomolecules such as glycan-binding proteins (GBP). Conventional methods such as isothermal titration calorimetry (ITC), surface plasmon resonance (SPR), and enzyme-linked lectin assay (ELLA) can be time consuming and require significant amounts of material.\textsuperscript{5–7} Moreover, multivalent interactions between carbohydrate ligands and GBPs are generally required to achieve good binding.

The emerging of glycan microarrays as high-throughput technology for studying carbohydrate interactions have overcome some of these challenges, and have greatly contributed to our understanding of the biological roles of carbohydrates or glycoconjugates and their interactions with a variety of macromolecules.\textsuperscript{8–13} Glycan microarrays allow for the rapid screening of thousands of binding interactions in one experiment using minimal amounts of precious material. Therefore, glycan microarray has become a powerful tool for studying carbohydrate protein interaction. Herein, we describe the use of glycan microarrays in biomedical applications and their potential role in drug discovery and development.
2 Fabrication of glycan microarrays

The key step in the fabrication of glycan microarrays involves the immobilization of the glycans into solid support. Various solid supports such as glass microscope slides, microtiter plates, gel beads, and nitrocellulose membranes have been utilized in the construction of glycan microarrays. The two main methods used for glycan array fabrications are non-covalent immobilization and covalent immobilization (Fig. 6.1). The most commonly used solid support is microscope glass slides due to their compatibility with optical detection systems, being relatively inexpensive, and ease of surface functionalization.

Non-covalent immobilization techniques are mainly through hydrophobic and electrostatic interactions. Free or modified glycans are absorbed into underivatized or derivatized solid surfaces. For example, polysaccharides and neoglycolipids have been passively adsorbed on nitrocellulose-coated glass slides.\(^{14-16}\) Negatively charged heparin polysaccharides have been immobilized into lysine-coated slides via electrostatic interactions.\(^{17}\) Alternative non-covalent immobilization method involves fluorphilic interaction between fluorous-tagged glycans and slides coated with fluoroalkylsilane.\(^{18,19}\) Moreover, other non-covalent immobilization such as using biotinylated sugars to streptavidin-coated surfaces and DNA-based glycan arrays have been reported.\(^{20-22}\)

Covalent immobilization of glycan arrays usually involves having reactive groups at the end of spacer moieties that reacted with functionalized surfaces to form a covalent bond. Covalent immobilization methods often involved amine and thiol chemistry. For example, amine-terminated glycans are immobilized into functionalized glass slide surfaces by reacting

![Figure 6.1 Glycan microarrays immobilization.](image)

(A) Noncovalent immobilization techniques based on hydrophobic absorption or by other interactions (electrostatic, fluorphilic, etc); (B) Covalent immobilization by linking a reactive group to the functionalized surface via (cycloaddition, epoxide opening, amide, thioether, etc).
with N-hydroxysuccinamide (NHS) activated ester, epoxide, or aldehyde via covalent bonds.\textsuperscript{23–26} Other glycan arrays have been constructed using thiol chemistry by reacting maleimide functional group via disulfide bond formation or reacting with gold surfaces.\textsuperscript{27–31} Other methods used to construct glycan arrays include cycloaddition reaction of azide with an alkyne, amine with epoxide functionalized surfaces, and free glycans with amino-oxy or hydrazide surfaces.\textsuperscript{32–35}

### 3 Detection of glycan microarrays

Various methods have been developed for glycan microarray detection including fluorescent-based methods, surface plasmon resonance, and mass spectrometry (Matrix-Assisted Laser Desorption/Ionization-Time of Flight, MALDI-TOF).\textsuperscript{36} Because of their high sensitivity and availability, fluorescent-based methods are the most commonly used methods for the detection of glycan microarray. Fluorophores such as fluorescein isothiocyanate, Cy3, and Cy5 are often coupled to glycan binding proteins (GBP) or secondary antibodies for detecting interactions with glycan microarrays. In general, detection of glycan microarray involves incubating the glycan array with GBPs, washing unbound proteins, subsequent incubation with detection reagents if necessary, followed by multiple washing steps after incubation, detection and quantification of the fluorescent signals (Fig. 6.2).

Figure 6.2 Fluorescent-based method for glycan microarray detection.
For glycan-binding molecules labeled with a fluorescent tag, read-out of the fluorescence intensities from the microarray can be obtained directly. For unlabeled GBP, a second incubation step with labeled detection reagents such as antibodies is commonly used. Alternatively, label-free detection methods, such as SPR and mass spectrometry have been employed to evaluate the interactions of GBPs. The use of gold surfaces as array support for immobilizing thiol-linked glycans allows the use of glycan arrays for SPR studies. Using multi-channel SPR instruments hundreds of glycans microspots can be analyzed simultaneously.\textsuperscript{37,38}

\section*{4 Biomedical applications of glycan microarrays}

Glycan microarrays have been used for studying the binding interactions of a variety of glycan-binding molecules such as proteins, antibodies, cells, bacteria, and viruses. In the past two decades, glycan microarrays have become the ideal format for studying carbohydrate protein interactions. Hundreds of glycans can be screened to study the binding interactions, activities, and specificities of various glycan-binding proteins. Therefore, glycan microarray has emerged as a powerful high-throughput tool for studying the biological role of glycans and as a potential tool for disease detection and vaccine development (Fig. 6.3). It has been utilized for the

\textbf{Figure 6.3} Biomedical applications of glycan microarray.
screening for biomarkers for a wide variety of diseases including cancer, infectious diseases, autoimmune diseases, and for monitoring immune response to vaccines. Additionally, glycan microarray can be employed in the high-throughput screening of potential new inhibitors for enzymes that are known to be complicated in the biosynthesis of disease-related glycans.

4.1 Cancer

Glycan microarrays have been used in evaluating cancer antigens as potential cancer biomarkers for cancer detection, prognosis, and response to treatment. Expression of altered glycans on cell surfaces is one of the hallmarks of cancer, and the alternation in glycosylation patterns can be explored for the diagnosis of cancer, drug targeting treatment, and differentiating cancerous cells from normal cells. For example, higher-ordered branching of N-linked glycans with an increased level of fucosylation and sialylation has been reported to be associated with cancer development. On the contrary, the structure of the O-linked glycans are often much more truncated. The use of these glycans as potential biomarkers has been extensively studied, but they are not ideal for the early detection of cancer as they are difficult to isolate from the tumor site. Alternatively, in the promise that aberrant glycosylation in tumor cells may give rise to changes in antibody levels aberrant glycans, many research groups have focused on profiling antibodies to tumor-associated carbohydrate antigens (TACAs) as potential biomarkers for the early detection, diagnosis, and prognosis of cancer.

Prior to the development of glycan microarray technology studies have focused on profiling antibodies to a very small number of available carbohydrate antigens, including Tn antigen, TF antigen, and some gangliosides. Glycan microarrays allow for the high throughput screening of hundreds of glycans using a miniscule amount of material. A number of groups have demonstrated the use of glycan arrays to identify anti-glycan antibodies in serum as potential biomarkers for the early detection, diagnosis, and prognosis of cancer. For example, glycan microarrays have been utilized to profile antibody levels to Globo H and related structures in patients with breast cancer. They reported significantly higher levels of antibodies against Globo H in patients versus the healthy control group ($P < 0.0001$).

From profiling antibodies of patients with breast cancer and healthy controls, Wandall et al. reported the presence of higher levels of antibodies to MUC1 in patients with breast cancer compared to that of healthy
The antibodies against MUC1 were specific to the glycan moiety and peptide sequence, and no cross-reactivity was observed with other glycopeptides containing the same glycan epitopes. In addition, the same group profiled antibodies in serum of patients with colorectal cancer and identified cancer-associated IgG and IgA antibodies to a set of altered MUC1 and MUC4 glycopeptides. The tumor-associated Tn and sialylated Tn glycans were the main glycan antigens associated with these epitopes. Moreover, the tumor-associated Tn and TF antigens have been greatly examined as potential diagnostic and prognostic biomarkers as well as therapeutic targets for cancer. Tn/TF antigen-based vaccines for prostate cancer and breast cancer were developed and have progressed into clinical trials. Blixt and co-workers also reported a significantly higher levels of antibodies to tumor-associated MUC1 in patients with early-stage breast cancer, but not in late-stage breast cancer patients. They reported that the levels of IgG antibodies to MUC1 carrying core 3 glycans and sialylated Tn antigens are associated with reduced incidence and delay in metastases, which suggest that these antibodies may play a role in the progression of cancer and can be used as potential prognostic biomarkers.

A study using glycan microarray for profiling antibodies of non-mucinous ovarian cancers reported that antibodies against a set of glycans can differentiate between non-mucinous borderline or ovarian cancer from healthy controls and that $P_1$ (Gal\(\alpha_1-4\)Gal\(\beta_1-4\)GlcNAc\(\beta\)) was the best candidate ($P < 0.001$) for detecting ovarian cancer. In another study, Vuskovic et al. used glycan microarray to profile antibodies in patients with mesothelioma and high-risk subjects that were exposed to asbestos. They reported that glycan arrays can be used for the diagnosis and prognosis of mesothelioma. The antigen Neu5Ac\(\alpha_2-3\)Gal\(\beta_1-4\)Glc\(\beta\) has the best correlation for diagnosis ($P < 0.0005$), while Glc\(\alpha_1-4\)Glc\(\beta\) showed the best correlation for prognosis ($P > 0.005$). A Comparison study of serum antibody levels in classical Hodgkin’s lymphoma and healthy controls demonstrated that antibody levels to GalNAc\(\alpha\)-Ser/Thr (Tn) were significantly higher in patients with classical Hodgkin’s lymphoma.

Several studies reported that dietary non-human glycans, such as $N$-glycolylneuraminic acid (Neu5Gc) incorporation into human tissue cell surfaces is associated with the development of cancer. Comparing the antibody profiles of patients with carcinomas and other diseases showed that antibodies to Neu5Gc\(\alpha_2-6\)Tn were prominent in patients with carcinomas. These antibodies can mediate the killing of Neu5Gc\(\alpha_2-6\)Tn–expressing tumors indicating that antibodies against Neu5Gc might serve as diagnostic
and prognostic biomarkers or as immunotherapeutic agents in human carcinomas. Additionally, antibodies to N-glycans such as high mannose and multi-antennary type II chains have been detected in human sera of patients with prostate cancer.

### 4.2 Infectious diseases

Many pathogens contain specific glycans on their cell surfaces that can elicit immune responses in infected individuals producing specific antibodies to the invading pathogens. Therefore, various glycan microarrays have been developed and used to evaluate immune responses to infectious diseases to detect pathogen-specific diagnostic biomarkers. For example, glycan microarray was used to profile the sera of individuals infected with the parasite, *T. spiralis* and reported that GalNAcβ1-4(Fucα1-3)GlcNAc (LDNF) antigen presented on BSA (bovine serum albumin) as a potential diagnostic biomarker for trichinellosis with very good sensitivity (96%) but modest specificity (67%). Seeberger and co-workers used glycan microarray to profile antibodies in malaria patients and healthy controls, and reported that exposure to malaria can affect the antibody levels to glycosylphosphatidylinositol (GPI) and their reactivity pattern control. They reported that the minimal epitope required for binding with anti-GPI antibodies is the pentasaccharide (Man₃-GPI, Manα1-2Manα1-6Manα1-4GlcNH₂α1-6-myoinositol-1-PO₄). Another study indicated that specific antibodies that recognize glycopeptides bearing the Tn antigen is potentially useful for the diagnosis of *Cryptosporidium parvum* infection.

Glycan microarrays have also been utilized to find biomarkers for viral infections. The initial step in viral cell invasion is the attachment of the virus to cell surface antigens that are often times glycans. The adhesion of the virus to the cell surface is mediated by viral surface proteins such as hemagglutinin (HA) in the case of influenza viruses. Glycan microarrays containing sialylated glycans have been used to profile the binding preferences of several human and avian hemagglutinins. These studies indicated that human H3N2 viruses preferentially bind to alpha2-6-linked N-acetylneuraminic acid (Neu5Acα2-6-linked) glycans, while the avian H5N1 viruses prefer binding to Neu5Acα2-3-linked glycans. In the recent outbreak of the H1N1 influenza virus in 2009, Tumpey’s and colleagues demonstrated that the virus exhibited a dose-dependent binding to only Neu5Acα2-6-diLacNAc (LacNAc = Galβ1-4GlcNAc) [69]. In another study, a sialylated glycan array was used to study the interactions of modified sialic acids using proteins and viruses. The results
demonstrated that influenza viruses H1N1 and H3N2 have preferences for binding to only the alpha2–6-linked glycans containing Neu5Ac or Neu5Ac9Lt (9-O-Lactoyl-N-acetylneuraminic acid). Moreover, Blixt and colleagues constructed a glycopeptide array to study the immune responses to the herpes simplex viruses (HSV-1 and HSV-2). They reported the presence of IgG antibodies to the glycopeptide P-P-A-(GalNAc)T-A-P-G in HSV-2 infected individuals but not in healthy controls, or those infected with HSV-1. Furthermore, glycan microarray has been used to detect anti α-fetoprotein fraction L3 (AFP-L3) for early prediction of Hepatitis B hepatocellular carcinoma. They demonstrated that AFP-L3 antibody levels is better for differentiating between hepatocellular carcinoma (HCC) and chronic hepatitis B (CHB) and could potentially be used as a biomarker for the early diagnosis of HCC.

Glycan microarrays have also been used to identify biomarkers in detecting and differentiating bacterial infections. Microarray platform containing polysaccharide has been constructed to identify potential markers for microbial infections by profiling antibodies in serum of infected human or animal subjects. Parthasarathy et. al. probed the use of polysaccharide array to detect antibodies against Francisellatularensis (causative agent of tularemia), Burkholderia pseudomallei (causative agent of melioidosis), and Bacillus anthracis (causative agent of anthrax). The results from this study demonstrated that glycan arrays can specifically detect and differentiate subjects infected with tularemia, melioidosis, and anthrax. Another study evaluated the use of lipopolysaccharide (LPS) for detecting antibodies in canine serum collected from tularemia positive and control subjects. The results indicated that LPS microarrays can detect anti-LPS antibodies at low concentrations and better sensitivity than the conventional immunofluorescence assays. Moreover, Blixt and co-workers used glycan microarray to profile antibodies in sera of patients diagnosed with salmonellosis and detected Salmonella specific antibodies in infected subjects. In addition to detecting anti-glycan antibodies in sera, glycan microarrays have also been used to study direct interactions of bacteria and array glycans. The results demonstrated that glycan microarrays can be used to discriminate different bacteria strains and that bacterial detection can be accomplished using complex mixtures. Using shotgun glycomics, Song et al. profiled sera from individuals infected with Lyme disease. The study showed that higher antibody levels to disialylated ganglioside (GD1b-lactone) were present in subjects with Lyme disease and can potentially be used as diagnostic markers for Lyme disease.
4.3 Autoimmune diseases

Autoimmune diseases arise from complex interactions of various factors such as genetic and environmental factors and affect millions of individuals worldwide. Autoimmune diseases occur when the immune system mistakenly attacks its own cells or healthy tissues. Diagnosis and prognosis of autoimmune diseases can be very challenging due to the lack of specific symptoms to a particular autoimmune disease. Human cells and a variety of macromolecules in nature are covered with dense complex glycans, also known as glycocalyx. These glycans play an important role in facilitating cell communications, pathogen recognition, and modulating both innate and adaptive immunity. Higher anti-glycan antibody levels in patients with autoimmune diseases are often correlated to disease progression. Therefore, anti-glycan antibodies can potentially be used in the detection and prognostic testing of autoimmune diseases.

The development of glycan microarray technology has allowed for the systematic screening of serum samples of patients with autoimmune diseases. This has led to the discovery of anti-glycan antibodies as potential diagnostic and prognostic markers for some autoimmune diseases such as Crohn’s disease (CD) and multiple sclerosis. Profiling anti-glycan antibodies in patients with chronic inflammatory bowel disease (IBD) has led to the identification of anti-glycan antibodies as biomarkers for CD. Dotan et al. reported the use of glycan arrays to identify novel anti-glycan antibodies against laminaribioside ($P < 0.001$) and chitobiosides ($P < 0.05$) that are specifically associated with CD. Antibodies against laminaribioside, chitobiosides, or mannan can be sued to differentiate CD patients from ulcerative colitis (UC) patients with over 99% specificity when two of these markers are combined. The results of this study were further validated using an enzyme-linked immunosorbent assay.

Multiple sclerosis (MS) is another autoimmune disease that affects the central nervous system as a result of damage to the myelin sheath. A number of research groups have attempted to identify potential biomarkers for the diagnosis and prognosis of MS. Miller and colleagues used glycan microarray to profile serum antibodies of patients with relapsing-remitting multiple sclerosis (RRMS). They reported significantly higher IgM anti-glycan antibodies to Glcα1-4Glcα ($P < 0.0001$) in patients with RRMS in comparison to patients suffering from other neurological diseases. The results from this study were further validated in a separate study using enzyme-linked immunoassay suggesting that anti-Glcα1-4Glcα IgM can...
be used as a potential biomarker for the diagnosis or prognosis of MS.\textsuperscript{85} Furthermore, Grader-Beck et al. reported the presence of distinct antibodies to 4-sulfated LacNAc \([4S-\text{LacNAc}, 4-(\text{OSO}_3)\text{Gal}\beta 1-4\text{GlcNAc}]\) in patients with systemic sclerosis \((P = 0.02)\).\textsuperscript{86} The presence of antibodies to 4S-LacNAc was also associated with a high prevalence of pulmonary hypertension. This suggests that post-glycosylation modifications of glycans can possibly be immunogenic and may play a key role in disease progression.

### 4.4 Vaccine development

Glycan microarrays are very useful tools for profiling immune responses induced by therapeutics glycan conjugate or glycan-based vaccines. Blixt and co-workers used glycan microarrays to monitor immune response to MUC1 conjugated vaccine in cancer patients and detect the presence of vaccine-induced antibodies to MUC1 glycopeptides.\textsuperscript{87} Reactive antibodies to Tn-MUC1 epitope were only present in vaccinated patients. Globo-H based vaccines have been developed and made it to clinical trials. A glycan microarray study indicated that antibody levels against Globo-H were higher in patients with breast cancer.\textsuperscript{23} Comparing immune response to Globo-H vaccine candidates in mice showed higher antibody levels for the glycoconjugates with enhanced specificity.\textsuperscript{88} Zhang et al. utilized glycan microarrays to evaluate serum antibodies in patients with advanced prostate cancer before vaccination and 2–3 months after vaccination with a poxvirus-based vaccine (PROSTVAC-VF).\textsuperscript{89} The results indicated changes in antibody levels to a number of antigens including forssman antigen and blood group A antigens. The PROSTVAC-VF vaccine has reached advanced stages in clinical trials.\textsuperscript{90–92} A follow-up study reported that the pre-vaccination IgM levels to blood group A trisaccharide may be used to predict survival for PROSTVAC-VF vaccine.\textsuperscript{93}

The development of vaccines against pathogens is an ongoing effort. For example, anthrax-producing bacterium (\textit{Bacillus anthracis}) contains a tetrasaccharide composed of three rhamnose and terminal anthrose on the spore surface of BC1A glycoprotein. Wang et al. used glycan microarrays to show that vaccine-induced rabbit anti-anthrax antibodies bind strongly to the trisaccharide \((\text{Ant}\beta 1-3-L\text{-Rhap}\alpha 1-3-L\text{-Rhap})\) and tetrasaccharides \((\text{Ant}\beta 1-3-L\text{-Rhap}\alpha 1-3-L\text{-Rhap}\alpha 1-2-L\text{-Rhap})\).\textsuperscript{94} The results illustrated that anthrose-containing oligosaccharides are immunogenic and are potential candidates for developing anthrax vaccines. Glycan microarrays were also utilized in monitoring immune responses to inactivated SARS-coronavirus (SARS-CoV) vaccine.\textsuperscript{95} They reported significant levels of IgG antibody
levels to the human serum glycoprotein asialo-orosomucoid, indicating that the vaccine can elicit a glycan-dependent autoimmune response. Moreover, glycan microarrays have been used to monitor immune responses in mice to a synthetic fragment of the capsular polysaccharide (PS-II) of Gram-positive bacteria *Clostridium difficile* that was conjugated to an immunogenic carrier protein. The vaccine-induced antibodies in mice that specifically interacted with the synthetic fragment of PS-II. The same array was utilized to detect IgA antibodies in the stool of infected hospital patients suggesting that the hexasaccharide fragment of PS-II is a key target of the immune response produced in infected subjects.

Viral envelope glycoproteins are known to mask the virus from the host immune system and are believed to play a key role in viral infections. Therefore, developing vaccines to fight viral infections is a continuing effort. For example, broadly neutralizing antibodies including 2G12 have shown anti–HIV activity and can protect against HIV infection in macaques. Glycan microarray containing high-mannose oligosaccharides revealed that 2G12 specifically binds high mannose *N*-linked glycans containing Manα1–2Man determinants including Man8 and Man9. Glycan microarrays were used to monitor immune responses to see if 2G12-like antibodies are produced in vaccinated animals. Rabbits immunized with a mutant strain of *Saccharomyces cerevisiae* induced antibodies that recognized a broad range of HIV-1 and SIV envelope glycoproteins. Additionally, rabbits immunized with *Saccharomyces cerevisiae* deficient with α1–3mannosyltransferase produced sera that contained antibodies that with similar specificity to that of 2G12. Another study evaluating anti-glycan antibody responses to an SIV vaccine and SIV infection demonstrate the potential use of glycan microarrays in vaccine development.

### 4.5 Enzyme inhibitors

Although it’s not well explored, another important application of glycan microarrays is for the screening of potential inhibitors for glycan–processing enzymes including those that are known to be complicated in the biosynthesis of disease-related glycans. For example, a microtiter array was used to screen for fucosyl transferase inhibitors. The reported four potential fucosyltransferase inhibitors with inhibition constants in the nanomolar range. Fucosyltransferases transfer fucose to sialylLacNAc to form sialyl Lewis X, which is an antigen that is involved in inflammation. Blixt et al. used glycan microarray to screen various recombinant sialyltransferase to determine acceptor specificities and made a series of unnatural sialosides.
5 Conclusions

Glycan microarray is a powerful and transformative high throughput tool for studying protein–carbohydrate interactions. It has been extensively used for study the binding interactions, activities, and specificities of various glycan-binding proteins. Glycan microarrays have been used in a variety of biomedical applications, however, the potential of this technology has not been optimized. This is in part due to the limited number of glycans available compare to the glycan diversity found in nature. For example, only small fractions of the human glycan repertoire are incorporated into the arrays. With developments of new chemical and enzymatic methods for the synthesis of complexed carbohydrates and more advances of current microarray technology, more applications will be explored.

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